CONTRACTILE AND BIOCHEMICAL ADAPTATION OF THE DIAPHRAGM TO ENDURANCE EXERCISE

By

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The roots of education are bitter, but the fruit is sweet.

[Aristotle. Apothegm]

This dissertation is dedicated to my parents Sotiris and Dimitra and my sister Lena, for creating the loving family environment I was brought up into.

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Abstract of Dissertation Presented to the Graduate School
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CONTRACTILE AND BIOCHEMICAL ADAPTATION OF THE DIAPHRAGM TO ENDURANCE EXERCISE

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Major Department: Exercise and Sport Sciences

Recent data from our laboratory demonstrate that endurance training increases the oxidative and antioxidant capacity of the rat costal diaphragm. There is also evidence that endurance training results in significant reduction in percent type IIb myosin heavy-chain (MHC) in costal diaphragm. At present, the effects of these biochemical and phenotypic alterations on the contractile and fatigue properties of the costal diaphragm are unknown. Therefore, the purpose of these experiments was to test the following hypotheses: 1) endurance training will reduce diaphragmatic rate of fatigue development, 2) endurance training will result in decreased diaphragmatic maximal shortening velocity (V_{max}) and therefore decreased maximal power output, and 3) endurance training will not effect diaphragmatic specific force. To test these hypotheses, isometric and isotonic contractile properties, as well as fatigue rate were measured *in vitro* on costal diaphragm strips from trained and untrained female Sprague-Dawley rats. Costal and crural diaphragm cytrate synthase (CS), superoxide dismutase (SOD), and glutathione peroxidase (GPX) activities, and costal diaphragm MHC and myosin light-chain (MLC) composition, were also measured. Two separate *in vitro* experiments were conducted, and a total of sixty-four animals were divided into two groups: exercise trained and control. The training program consisted of running on a motor driven treadmill five days per week, with a duration of sixty minutes per day and an intensity of ~75% of VO_{2max}, over a ten week period. Results revealed that exercise had a fatigue sparing effect on the trained diaphragms. Control costal diaphragm strips produced an average of 26% of their initial force during the last thirty minutes of the *in vitro* fatigue protocol, where as the force production of the trained diaphragm strips remained significantly ($P<0.01$) higher at 31.2%. This effect was accompanied by 10.1% CS and 12.1% SOD higher ($P< 0.05$) activities in the trained diaphragms compared with the controls. Despite the decrease ($P< 0.05$) in type IIb MHC (7.2±1.5 vs 10.9±1.0%) and the increase ($P< 0.05$) in type I MHC (36.1±0.7 vs 32.7±0.8%) in trained diaphragms, diaphragmatic V_{max} was not different between trained (6.35 ± 0.13 lengths • sec^{-1}) and control (6.57 ± 0.20 lengths • sec^{-1}) animals. No differences were observed in the relative proportions of MLC between groups. Further, results indicated that diaphragmatic maximal tetanic force (P_o) normalized to strip CSA was not different between the trained (25.52±0.4 N • cm^{-2}) and control diaphragms (24.32±0.5 N • cm^{-2}). These data support the hypothesis that endurance training reduces the rate of diaphragmatic fatigue *in vitro*. The hypothesis that endurance training results in decreased diaphragmatic V_{max} and therefore decreased maximal power output was not supported from these results. Finally, the data support the hypothesis that endurance training does not effect diaphragmatic specific force.

CHAPTER 1
INTRODUCTION

The diaphragm is the most important inspiratory muscle in mammals. It is chronically active, and is the only skeletal muscle that can be considered a vital organ. It's primary function is to increase the dimensions of the chest cavity and thus exchange lung gas to maintain arterial blood homeostasis. Inability to do so, due to diaphragmatic failure as a result of fatigue or disease, could have lethal consequences. Heavy prolonged exercise has been shown to impair respiratory muscle performance in humans (Aaron *et al.*, 1985; Babcock *et al.*, 1995a; Babcock *et al.*, 1995b; Coast *et al.*, 1990; Johnson *et al.*, 1993; Mador *et al.*, 1993). Further, patients with chronic obstructive lung disease often exhibit reduced respiratory endurance (Rochester and Arora, 1983; Roussos and Macklem, 1982). Therefore, improving our understanding of the cellular adaptation of respiratory muscles may lead to important clinical benefits in the management of patients with lung disease or other disorders that might cause respiratory muscle fatigue. Hence, the study of diaphragmatic adaptation to endurance exercise is of paramount importance.

Until the late 1980s the issue of whether endurance training increases the oxidative capacity of the diaphragm was controversial (Faulkner *et al.*, 1972; Fregosi *et al.*, 1987; Green and Reichmann, 1988; Ianuzzo *et al.*, 1982; Lieberman *et al.*, 1972; Metzger and Fitts, 1986; Moore and Gollnick 1982). A series of rat training experiments have been conducted since, to resolve the controversy. It is well established today, that endurance training results in significant increases (20-

30%) in mitochondrial enzyme activity within the costal diaphragm (Gosselin *et al.*, 1992a; Grinton *et al.*,1992; Lawler *et al.*, 1993; Powers *et al.*, 1992b; Powers *et al.*, 1994a; Powers *et al.*, 1992c; Powers *et al.*, 1992e; Powers *et al.*, 1990b; Sugiura *et al.*, 1992a; Sugiura *et al.*, 1990; Uribe *et al.*, 1992).

Recent studies in our laboratory have also demonstrated that endurance exercise training results in significant increases in the activities of the costal diaphragm antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPX) (Powers *et al.*, 1994a). Similar findings have been reported in locomotor muscles (Criswell *et al.*, 1993; Higuchi *et al.*, 1985; Laughlin *et al.*, 1990; Powers *et al.*, 1994b). In addition to the above adaptations of the diaphragm to endurance training, several authors have noted a decrease in the cross-sectional area (CSA) of costal diaphragmatic fibers with no change in the capillary-to-fiber ratio (Gosselin *et al.*, 1992a; Green *et al.*, 1987; Green *et al.*, 1989; Powers *et al.*, 1992a; Powers *et al.*, 1992b; Tamaki 1987).

Theoretically, all of the aforementioned adaptations of the costal diaphragm to exercise training would improve endurance and delay fatigue. An increase in oxidative capacity of the tissue would enhance fat utilization, reduce the rate of glycogen degradation and decrease the rate of lactate production (Gollnick *et al.*, 1985; Holloszy, 1967; Holloszy and Coyle 1984). An increase in antioxidant enzyme activity would enable the diaphragm to more efficiently eliminate the free radicals that formed during the increased mitochondrial respiration caused by exercise, by acting directly on fatiguing myocytes (Khawli and Reid 1994). This is important since free radical formation has been shown to promote fatigue, and exogenous antioxidants slow the fall of force output in fatiguing muscle (Barclay and Hansel, 1991; Diaz *et al.*, 1992; Reid *et al.*, 1992a; Shindoh *et al.*, 1990; Supinski *et al.*, 1991). Finally a decrease in the CSA of costal diaphragmatic fibers with no change in the capillary-to-fiber ratio would improve the fiber's relative oxidative function (Powers *et al.*, 1992a; Powers *et al.*, 1992b), as well as, reduce the distance for diffusion of gases, metabolites, and/or substrates (Enad *et al.*, 1989). Several studies have demonstrated that respiratory muscle training in humans results in improvement in ventilatory endurance (Levine *et al.*, 1986; O'Kroy and Coast 1993). Unfortunately, similar data on animals are not available.

There is also evidence that endurance training results in a significant reduction in percent type IIb myosin heavy-chain (MHC) in costal diaphragm (Sugiura *et al.*, 1990; Sugiura *et al.*, 1992a). In rat skeletal muscle, fiber maximal shortening velocity (V_{max}) is higher in the type IIb fibers compared to the type I, while the V_{max} in the type IIa and IIdx fibers is intermediate (Gardetto *et al.*, 1989). In theory, a shift in MHC isoforms would affect muscle V_{max}. It has been shown that endurance swim training in man results in a decreased V_{max} of the fast fibers of the deltoid (Fitts *et al.*, 1989).

At present, only one report has examined the effects of endurance training on the contractile properties and fatigue of the diaphragm (Metzger and Fitts 1986). These authors reported that training had no effect on specific tension, V_{max},or fatigue rate of the costal and crural diaphragm. However, their training protocol did not induce any increases in the oxidative capacity of the diaphragm and most likely it did not affect antioxidant enzyme activities or MHC isoforms. Hence, there is a clear need for additional work in this area.

Statement of Problem

It has been shown that endurance training increases oxidative and antioxidant capacity of the costal diaphragm as well as capillary density. A fast to slow shift of the MHC isoforms of the diaphragm has also been noted to follow

low intensity endurance exercise. The physiological effects of these adaptations are not known today. The present study was designed to systematically examine the effects of these exercise induced cellular diaphragmatic adaptations in the fatigue rate, V_{max}, and specific force of the rat costal diaphragm.

Research Hypotheses

Based on the ideas discussed above these experiments were designed to test the following hypotheses:

1) Question

Does endurance training result in improved diaphragmatic endurance?

Hypothesis

Endurance training will reduce the rate of diaphragmatic fatigue development measured *in vitro*.

2) Question

Does endurance training result in decreased diaphragmatic maximal shortening velocity and maximal power output?

Hypothesis

Endurance training will result in decreased diaphragmatic maximal shortening velocity and therefore maximal power output will decrease.

3) Question

Does endurance training result in decreased diaphragmatic specific force?

Hypothesis

Endurance training will not affect diaphragmatic specific force.

Definition of Terms

1/2 RT: Relaxation half-time following a twitch contraction.

A/D: Analog to digital.

a/Po: Curvature of the force-velocity relationship, where a = a constant derived from the hill equation.

α: Level of significance.

CAT: Catalase converts hydrogen peroxide to water and O_2.

CSA: Cross sectional area.

CS: Citrate synthase. Kreb's cycle enzyme located in the mitochondria. Catalyzes the initial reaction of the cycle that begins with the condensation of acetyl-CoA with oxaloacetate to give citrate. Used as a marker of the oxidative capacity of the diaphragm and plantaris muscles.

dP/Dt: Maximal rate of specific force development during a twitch contraction.

EC: Excitation and contraction coupling.

GPX: Glutathione peroxidase acting with reduced glutathione can reduce hydroperoxides to form alcohol or water.

H_2O_2: Hydrogen peroxide is a product of the dismutation of superoxide by superoxide dismutase.

L_o: Optimal length.

MHC: Myosin heavy chain.

MLC: Myosin light chain.

P: Level of significance.

P_o: Isometric tetanic specific force.

P_t: Isometric twitch specific force.

SDH: Kreb's cycle enzyme located in the mitochondria. Succinate dehydrogenase catalyzes the first of the three reactions involved in the completion of the cycle converting succinate to oxaloacetate.

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

SOD units: One unit is equal to the amount of activity which causes 50% inhibition of Epinephrine oxidation.

SOD: Superoxide dismutase catalyzes the dismutation of superoxide to O_2 and hydrogen peroxide.

SR: Sarcoplasmic reticulum.

V_{max}: Maximal shortening velocity.

Assumptions

Costal diaphragm strip CSA was calculated using the following formula, assuming that muscle density equals 1.056 g/cm^3 (Metzger and Fitts, 1986): CSA (cm^2) = muscle mass (g) / [muscle length (cm) x muscle density (g/cm^3)].

Limitations

An important determinant of the use of the *in vitro* preparation as a model for evaluating contractile properties of diaphragm fiber bundles, is that the stimulus used to excite the muscle studied, is supramaximal. This ensures that all the muscle fibers are recruited, if valid comparisons are to be made between different treatments. We postulated that fast fatiguable fibers are responsible for the initial decrease in force production during fatigue, where fatigue resistant fibers play a later role. Our treatment (endurance exercise), affected predominantly the type I and IIa muscle fibers (Powers *et al.*, 1992a; Powers *et* *al*., 1992b). It is therefore difficult to study the effects of training induced increase in oxidative and antioxidant capacity on the fatigue rate of the diaphragm, by using a short duration fatigue protocol, since *in vitro* all muscle fibers are maximally recruited simultaneously, which is not the case *in vivo*. By extending our fatigue protocol beyond the point of ten minutes, we compared the fatiguability of these fibers (I and IIa) that were adapted to the stress of exercise. The results of the sixty minute fatigue supports our postulate.

Significance

The diaphragm is the most important inspiratory muscle in mammals, and is the only skeletal muscle that can be considered a vital organ. It's primary responsibility is to exchange lung gas, and thus, to maintain arterial blood homeostasis. Inability to do so, due to diaphragmatic failure as a result of fatigue or disease, could have detrimental consequences. It is clear that respiratory muscle fatigue, and particularly diaphragmatic fatigue, contributes to the development of respiratory failure in adult and newborn humans, as well as in experimental animals. Therefore, improving our understanding of the cellular adaptive strategies of the diaphragm, will expand our knowledge and may lead to important clinical benefits in the management of patients with lung disease or other disorders that might cause diaphragmatic fatigue.

CHAPTER 2
REVIEW OF RELATED LITERATURE

Introduction

It is clear that respiratory muscle fatigue, and particularly diaphragmatic fatigue, contributes to the development of respiratory failure in adult and newborn humans, as well as in experimental animals. Therefore, improving our understanding of the cellular adaptive strategies of the respiratory muscles may lead to important clinical benefits in the management of patients with lung disease or other disorders that might cause respiratory muscle weakness. Thus, numerous investigators have examined the adaptive strategies of rodent respiratory muscles in response to endurance exercise.

This review will discuss the cellular adaptations of the major respiratory muscles to endurance training, with special emphasis on the costal diaphragm, as well as, the physiological significance of such adaptations. Further, this review will examine the possible mechanisms of respiratory muscle fatigue. I begin with an overview of the functional anatomy of the major respiratory muscles.

Functional Anatomy of the Respiratory Muscles

Inspiratory Muscles

Any muscle that increases the dimensions of the chest wall is an inspiratory muscle. The diaphragm, parasternal intercostals, external intercostals, scalenes, and sternocleidomastoids are considered as the important muscles of inspiration in mammals (De Troyer and Estenne, 1988). Of these inspiratory muscles, the three that have received significant experimental attention in regard to exercise adaptation are the diaphragm, parasternal intercostals, and external intercostals.

The diaphragm is a musculotendinous sheet that separates the thoracic cavity from the abdominal cavity and is the most important inspiratory muscle in mammals. Functionally and metabolically the diaphragm can be classified as two muscles: 1) the crural (dorsal) portion that inserts into the lumbar vertebrae; and 2) the costal portion that inserts into the hyphoid process of the sternum and into the margins of the lower ribs. These two areas have different ventilatory responsibilities (DeTroyer *et al.*, 1982; DeTroyer *et al.*, 1981; DeTroyer and Estenne, 1981). Further, costal diaphragm has higher oxidative capacity (18% greater), as well as protein concentration compared with the crural portion of the diaphragm (Powers *et al.*, 1990a; Powers *et al.*, 1991). Moreover, the crural diaphragm contains less type I MHC and more type IIb MHC compared to the costal (Sugiura *et al.*, 1992b). It is therefore important to consider the crural and costal diaphragm regions as separate muscles, when examining the adaptations of the diaphragm to exercise training.

The inspiratory action of the diaphragm on the rib cage has two components. First, during inspiration the rise in intraabdominal pressure is transmitted through the apposed diaphragm to expand the lower rib cage. This is termed the "apositional" component. Second, when the diaphragm contracts, during inspiration, pushes against the solid abdominal visceral mass to lift the lower ribs by way of its insertions. This is termed the "insertional" component.

The parasternal muscles consist of the interchondral portion of the internal intercostals. Because of the orientation and insertions of their muscle fibers, when the parasternals contract they produce a rotation of the chondrosternal junctions which results in an elevation of the ribs and a descent of the sternum and thus they considered as important inspiratory muscles.

The external intercostal muscles are superficial to the internal and extend from the articulations between the ribs and vertebral bodies to the origin of the costal cartilages. The lower insertion of the fibers of the external intercostals is more distant from the center of rotation of the ribs than the upper insertion. Therefore, when the muscle contracts, the torque acting on the lower rib is greater than that acting on the upper rib, and the net result is to raise the ribs, which is an inspiratory action.

Expiratory Muscles

Expiratory muscles act to decrease the dimensions of the chest wall and thus produce expiration. The internal intercostals, triangularis sterni, and the abdominals, are considered as the important muscles of expiration in mammals (De Troyer and Estenne, 1988). Of these expiratory muscles, those that have received significant experimental attention in regard to exercise adaptation are the internal intercostals, and abdominal muscles.

The internal intercostal muscles extend from the sternum to the angles of the ribs. The lower insertion of the fibers of the internal intercostals is less distant from the center of rotation of the ribs than the upper insertion. Therefore, when the muscle contracts, the torque acting on the upper rib is greater than that acting on the lower rib, and the net result is to lower the ribs, which is an expiratory action.

The abdominal muscles comprise the ventrolateral wall of the abdomen. The rectus abdominis originates from the cartilage of the fifth, sixth and seven ribs and xiphoid process and inserts into the pubis. The external oblique originates from the external surface of the lower eight ribs and inserts to the linea alba. The internal oblique originates from the iliac crest and inguinal ligament and insert to the cartilage of the last three or four ribs. The transversus abdominis originates from the iliac crest, inguinal ligament, lumbar fascia, and the lower six ribs and inserts into the linea alba and pubis. These four muscles rotate and flex the trunk. As respiratory muscles, they have two principal actions. First, when they contract, they pull the abdominal wall inward and thus increase intraabdominal pressure which causes the diaphragm to move cranially into the thoracic cavity and hence decrease lung volume. Second, they act to pull the lower ribs down and inward, which is an expiratory action.

Effects of Exercise Training on Respiratory Muscles

Oxidative Enzyme Changes

Until the late 1980's evidence on the metabolic plasticity of the rodent costal diaphragm, was equivocal. The findings from studies examining the notion that endurance exercise provides a sufficient stimulus to increase the muscle's oxidative capacity was controversial. The first studies to examine the effects of exercise training on the metabolic properties of the costal diaphragm, utilized guinea pigs trained on a motor driven treadmill for 8 weeks (Lieberman *et al.*, 1972; Faulkner *et al.*, 1972). The authors concluded that endurance exercise results in a 13-20% increase in the number of highly oxidative fibers in the costal diaphragm. These investigators used succinate dehydrogenase (SDH) as a mitochondrial marker enzyme for the histochemical analysis of the costal diaphragm cross-sections. Similarly, Ianuzzo *et al.* (1982), and Moore and Gollnick (1982) reported that treadmill exercise increased tricarboxylic acid cycle marker enzyme activity in the rat costal diaphragm by approximately 20-30%.

Studies reporting that endurance exercise training does not improve the oxidative capacity of the rat costal diaphragm challenged the above aforementioned findings. Metzger and Fitts (1986), reported no significant differences in the mitochondrial marker enzyme citrate synthase (CS) in the costal or crural regions of the diaphragm between high intensity trained and control animals. Fregosi *et al.*, (1987) also reported that substantial endurance training, inducing alterations in hole animal and plantaris muscle, is not sufficient stimuli to induce mitochondrial changes in the diaphragm. Green and Reichman (1988) confirmed these findings and reported that a prolonged (15 weeks) endurance training program significantly reduced CS and did not change β-oxidation enzymes. In an effort to resolve this issue, a series of experiments have been conducted in our laboratory (Grinton *et al.*, 1992; Lawler *et al.*, 1993; Powers *et al.*, 1992b; Powers *et al.*, 1994a; Powers *et al.*, 1992c; Powers *et al.*, 1992e; Powers *et al.*, 1990b). A wide variety of training protocols were employed including, exercise durations of 30 to 90 minutes, intensities of ~55% VO_{2max} to ~75-80% VO_{2max}, with continuous or interval treadmill running. Each of these studies demonstrated that endurance training results in a 20-30% increase in mitochondrial enzyme activity within the costal diaphragm in rats. Other laboratories confirmed these results. Gosselin *et al.* (1992), reported that SDH activity was significantly higher in I, IIa, and IIb costal diaphragmatic fibers in endurance trained animals compared with their sedentary counterparts. Uribe *et al.* (1992) showed that CS activity of the costal diaphragm was 20% greater in the trained animals. Sugiura *et al.*, in two separate studies (1990; 1992), reported that treadmill and swim training increased the aerobic capacity of the costal diaphragm. Therefore, it is clear that endurance training results in small but significant increases in mitochondrial enzyme activity within the costal diaphragm.

Recent evidence from our laboratory (Powers *et al.*, 1994a) demonstrates that endurance exercise training up-regulates the oxidative capacity (CS) of the crural diaphragm, if the training program is of high intensity and long duration. In this study 90 minutes of moderate or high intensity exercise was required to stimulate enough motor units within the crural diaphragm to promote a training adaptation. The same study demonstrated that the parasternal intercostals CS activity increased, as a function of both intensity and duration, by 40% after 10 weeks of endurance running.

Green and Reichman (1988) examined the effects of extreme endurance exercise on oxidative enzymes of the internal and external intercostals. In the external intercostal muscle CS and HAD increased by a range of 57-77%, whereas in the internal intercostal muscles no training induced alteration was evident.

Several investigators have studied the effects of endurance training on the oxidative capacity of the abdominal muscles (Grinton 1992; Powers 1992e; Uribe 1992). The results of these studies demonstrated that endurance training results in small but significant increases in mitochondrial enzyme activity of the rectus abdominis and external obliques. In contrast, Uribe (1992) showed that exercise training has no influence on the oxidative capacity of the internal obliques and transverse abdominis. The reason(s) for this discrepancy are unclear.

Respiratory vs. locomotor muscle

The magnitude of the training-induced alterations in oxidative capacity of the respiratory muscles (20-30%) is smaller compared to that of locomotor muscles (40-80%). At least two possible explanations exist. First, it is possible that exercise training places a smaller metabolic load on the diaphragm compared to locomotor muscles. Second, the basal mitochondrial enzyme activity in the costal diaphragm is significantly higher than a similar fiber composition locomotor muscle prior to training (Farkas and Roussos, 1982). Simoneau *et al.*, (1988) demonstrated that the magnitude of activity-induced oxidative changes of skeletal muscle is inversely related to the basal levels of enzyme activity.

Physiological significance of altered oxidative capacity

An increase in the oxidative capacity of the tissue would enhance fat utilization, reduce the rate of glycogen degradation and decrease the rate of lactate production (Gollnick *et al.*, 1985; Holloszy, 1967; Holloszy and Coyle 1984). In tasks that require prolonged endurance, glycogen depletion in the muscle has been correlated with muscle fatigue (Bergstrom and Hultman, 1967). The ability to spare glycogen and utilize lipids has been found to be associated with prolonged endurance (Hermansen *et al.*, 1967). Overall, the aforementioned tissue changes may delay fatigue during periods of high intensity contractile activity (Holloszy and Coyle 1984). This notion is supported by the significant correlation between diaphragm motor unit fatigue resistance and the mean SDH activity of muscle unit fibers, that was reported by Enad *et al.*, (1989). As mentioned earlier however, the training induced improvements in oxidative capacity in the costal diaphragm in rats are significant, but smaller than these reported for locomotor muscles.

Therefore a key purpose of this study is to determine if the aforementioned exercise training-induced improvements in oxidative capacity of the costal diaphragm, are of sufficient magnitude to significantly improve the muscle's fatigue rate *in vitro*.

Glycolytic Enzyme Changes

In contrast to mitochondrial enzyme adaptations to endurance training, costal diaphragm glycolytic enzymes appear not to be effected. Hexokinase is a possible exception. Ianuzzo *et al.*, (1982) showed that 12-16 weeks of treadmill running resulted in a 20% increase in hexokinase activity within the costal diaphragm. Powers *et al.*, (1992e) demonstrated that LDH activity within the costal diaphragm tends to decrease with endurance training. At present it is unclear if endurance training results in shifts in the LDH isoforms in the diaphragm.

The effects of endurance exercise on glycolytic enzymes in the crural diaphragm, parasternal muscles and external intercostals as well as in expiratory muscles, is currently unknown due to limited data. Green and Reichman (1988) examined the effects of extreme endurance exercise on glycolytic enzymes of the internal and external intercostals and reported that no training induced alteration was evident.

Antioxidant Enzyme Changes

Free radicals are unstable molecules or fragments of molecules with unpaired electrons in their outer orbital. Free radicals combine with electrons with opposite spins in other substances, to balance their unpaired electrons, and are therefore extremely reactive. Due to this extreme reactivity, free radicals are known to cause cellular dysfunction by poisoning enzymes, altering DNA, releasing destructive proteolitic enzymes, producing toxic biochemicals, and causing peroxidase chain reactions that unravel biomembranes (Sjodin *et al.*, 1990). Generation of ATP via oxidative phosphorylation results in formation of free radicals and hydroperoxides (Packer, 1986) and free radical production has been shown to be increased during exercise (Davies, 1982). Evidence exists to implicate free radical formation with exercise induced muscle fatigue and muscle injury (Davies, 1982; Starnes, 1989). Given the potential role of these reactive species in mediating muscular dysfunction, it is not surprising that cells contain several naturally occurring defense mechanisms to prevent oxidative injury. These protective mechanisms include the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). SOD catalyzes the dismutation of superoxide to O_2 and hydrogen peroxide (H_2O_2); H_2O_2 can be converted to water and O_2 by CAT. Furthermore, GPX acting with reduced glutathione can reduce hydroperoxides to form alcohol or water (Ji, 1995). Increasing the cellular activity of one or more of these antioxidant enzymes will reduce the risk of cellular injury from free radicals (Halliwell *et al.*, 1989).

Several investigators have reported that endurance exercise training up-regulates SOD and GPX activity in hindlimb muscles without altering CAT activity (Criswell *et al.*, 1993; Higuchi *et al.*, 1985; Laughlin *et al.*, 1990; Powers *et al.*, 1994b). Similar to these findings, recent studies in our laboratory have demonstrated that endurance exercise training results in significant increases in costal diaphragm SOD and GPX activities with no change in CAT activity (Powers *et al.*, 1994a). The same study demonstrates that high intensity training results in significant increases in crural and parasternal SOD activity with no change in GPX activity. The differential response of the two enzymes is not well understood.

Physiological significance of altered antioxidant enzyme activity

In theory an exercise-training -induced up regulation of SOD and GPX activity in the costal diaphragm would enable the diaphragm to more efficiently eliminate the free radicals that formed during exercise due to an increased mitochondrial respiration. Free radical formation has been shown to promote fatigue, and exogenous antioxidants have been shown to slow the fall of force output in fatiguing muscle (Barclay and Hansel, 1991; Diaz and Clanton, 1992; Shindoh *et al.*, 1990; Reid *et al.*, 1992a; Supinski *et al.*, 1991).

Shindoh *et al.*, (1990) reported that intravenous administration of N-acetylcysteine protected rabbit diaphragm against fatigue *in situ*. Khawli and Reid, (1994) tested N-acetylcysteine effects on fatigue of curarized directly activated fiber bundles *in vitro* and showed that this antioxidant is acting directly on skeletal muscle myocytes to inhibit fatigue. Other antioxidants that inhibit fatigue include superoxide dismutase, catalase (Reid *et al.*, 1992a), dimethyl sulfoxide (Barclay and Hansel, 1991; Reid *et al.*, 1992a; Supinski *et al.*, 1991), desferoxamine, and allopurinol (Barclay and Hansel, 1991). A possible mechanism of action for the resulting reduction of oxygen free radicals due to an increase in the antioxidant enzymes, may be the attenuation of the changes in the functional properties of the SR that accompanies skeletal muscle fatigue and which affect Ca^{2+} release and uptake. Khawli and Reid (1994), postulated that antioxidants reverse the process of sulfhydryl oxidation which promotes calcium release from the SR by opening release channels and inhibiting re-uptake pumps. An inhibition of free radical accumulation due to antioxidants would promote calcium sequestration by closing release channels and stimulate re-uptake enabling therefore a better calcium homeostasis. Whether or not the magnitude of the training-induced increase in SOD and GPX activity would improve diaphragmatic endurance *in vitro* is not clear and the proposed experiments aim to test this hypothesis.

Myosin Isoform Expression Changes

Investigations that used ATPase histochemistry to examine training-induced changes in diaphragmatic fiber type do not report exercise induced shifts in fiber type (Powers *et al.*, 1992a; Powers *et al.*, 1992b). However, recent studies have demonstrated that low intensity endurance running resulted in a significant decrease in the percentage of MHC IIb in the costal diaphragm (Sugiura *et al.*, 1992a). Low to moderate intensity endurance swimming also induced a decrease in the percentage of MHC IIb in the costal diaphragm (Sugiura *et al.*, 1990). Only two studies have examined the effects of endurance training on the MHC composition in the crural diaphragm (Gosselin *et al.*, 1992; Sugiura *et al.*, 1992a). Both investigators concluded that moderate intensity endurance training does not alter the MHC profile in the crural diaphragm. Finally, to date, no data exist concerning the effects of exercise training on parasternal and expiratory muscle myosin phenotype. In theory a shift in MHC isoforms would affect muscle maximal shortening velocity. It has been shown that endurance swim training in man decreased the Vmax of the fast fibers of the deltoid (Fitts *et al.*, 1989). Whether or not the magnitude of the training -induced decrease in MHC IIb in the costal diaphragm would alter diaphragmatic shortening velocity is not clear and the proposed experiments aim to examine this issue.

Contractile Changes

To date there is only one study (Metzger and Fitts 1986) that examined the effects of endurance training on the contractile properties and fatigue of the diaphragm. These authors reported that training had no effect on specific tension, V_{max}, or fatigue rate of the costal and crural diaphragm. However the training stimulus that was used in this study, was insufficient to induce increases in the oxidative capacity of the diaphragm and most likely, it didn't affect antioxidant enzyme activity and myosin heavy-chain isoforms. Since the diaphragm has higher mitochondrial capacity than locomotor muscles and since exercise constitutes a lower metabolic load for the diaphragm compared to locomotor muscle, a threshold of exercise intensity is essential to induce cellular adaptations (see above, respiratory versus locomotor muscle).

It is not surprising therefore, that the comparison of diaphragms with no biochemical, morphological or phenotypic difference would not reveal contractile differences. It was obvious to us, that the question of whether endurance exercise alters contractile properties and fatigue rate of the diaphragm needed to be answered by comparing control and trained diaphragms that exibid the cellular adaptations that have been recently documented to occur with endurance training.

Fiber Type Specific Adaptation of the Diaphragm

Several studies in our laboratory (Powers *et al.*, 1992a; Powers *et al.*, 1992) have examined the effects of endurance training on the oxidative capacity of the costal diaphragm, and reported that a reduction in fiber CSA and not an increase in the absolute fiber SDH activity, explained most of the increase in relative SDH activity within the costal diaphragm. In one of these experiments, the mean increase in relative SDH activity was 16% in type I and 18% in type IIa fibers in trained animals. However, training resulted in a reduction of ~10% in type I and 15% in type IIa fiber CSA. Therefore, the increase in total SDH activity in both type fibers was less than 5%.

Several other authors have reported that endurance training results in a decrease in the CSA of costal diaphragmatic fibers with no change in the capillary to fiber ratio (Gosselin *et al.*, 1992; Green *et al.*, 1987; Green *et al.*, 1989; Tamaki 1987).

Physiological significance of altered fiber CSA

A decrease in the CSA of costal diaphragmatic fibers with no change in the capillary-to-fiber ratio would improve the fiber's relative oxidative function, as well as, reduce the distance for diffusion of gases, metabolites, and/or substrates. This notion is supported by studies of the adult cat diaphragm by Enad *et al.* (1989). These authors reported that the more fatigable diaphragm motor units, were composed of fibers with larger CSA and lower SDH activities. In contrast, fatigue resistant units, were composed of smaller fibers with higher SDH activities. The average fiber size of muscle units were correlated with their fatigability.

Diaphragmatic Fatigue

Fatigue is defined as an inability to maintain a power output or force during repeated muscle contractions. The fact that the respiratory muscles may fail in humans was recognized in the late 1970s. At that time Roussos and Macklem (1977) reported that diaphragmatic fatigue can be induced in healthy humans in response to loaded breathing. Since then, a plethora of studies have been conducted, and it is now clear that respiratory muscle fatigue, contributes to the development of respiratory failure in humans (Muller *et al.*, 1979; Lopes *et al.*, 1981) and animals (Aubier *et al.*, 1981; Bazzy and Haddad 1984). Heavy prolonged exercise has been shown to impair respiratory muscle performance in humans (Aaron *et al.*, 1985; Coast, 1990; Johnson *et al.*, 1993; Mador *et al.*, 1993;

Babcock *et al.*, 1995a; Babcock *et al.*, 1995b). Further, patients with chronic obstructive lung disease often exhibit reduced respiratory endurance (Rochester and Arora, 1983; Roussos and Macklem, 1982).

Sites of Fatigue

The precise mechanisms that mediate skeletal muscle fatigue have yet to be identified, however it probably involves several factors that influence force production dependent on muscle fiber type and activation pattern (Williams and Klug, 1995). Possible sites of fatigue include activation at the central command, activation of the α-motor neuron and conduction of action potentials down to the neuromuscular junction, communication across the junction, and the coupling of excitation and contraction (EC) within the muscle fiber (Westerblad *et al.*, 1991). Investigators that attempted to deduce which of the aforementioned sites is responsible for diaphragmatic fatigue, by correlating changes in the phrenic neurogram with changes in the diaphragm electromyogram during loaded breathing, reported controversial findings (Aldrich *et al.*, 1986; Aldrich 1987; Aubier *et al.*, 1981; Watchko *et al.*, 1986; Watchko *et al.*, 1987). The various studies do not agree on the relative contribution of neural transmission failure versus contractile failure, however, it is generally accepted that the later plays a major role in diaphragmatic fatigue.

Alterations in Sarcoplasmic Reticulum Function and Ca^{2+} Handling

The notion that the decline in force output that accompanies fatigue involves changes in the EC processes within the muscle fiber was first raised by Eberstein and Sandow (1976). These investigators showed that reduction in force production during fatigue could be reversed by the application of caffeine, which activates the Ca^{2+} release channels. These results have been reproduced since then (i.e. Williams and Ward, 1991), and suggested an alteration in SR function of fatigued muscle that results in a reduction in the intracellular levels of Ca^{2+} due to a diminished SR Ca^{2+} release and/or uptake. There is evidence that SR function and thus Ca^{2+} handling are affected following exercise leading to fatigue (Fitts *et al.*, 1982; Belcastro *et al.*, 1981; Bonner *et al.*, 1976; Fitts *et al.*, 1979; Heilmann and Pette, 1979; Sembrowich and Gollnick, 1977; Byrd *et al.*, 1989; McCutcheon *et al.*, 1992).

Structural Alterations and Ca^{2+} Handling

It has been proposed that alterations in membrane lipid characteristics, due to changes in temperature associated with exercise, may influence the structure and function of the Ca^{2+} ATPase protein resulting in an altered rate of Ca^{2+} sequestration and ATP hydrolysis (Williams and Klug, 1995). Data from Henry and Klug (1995), supports this notion suggesting that muscle temperature may be a critical variable in the regulation of SR $Ca^{2+}Mg^{2+}$ -ATPase activity. There is also evidence that Ca^{2+} release by the SR of fatigued muscle is altered due to reduced number of functional channels responding to Ca^{2+} (Favero *et al.*, 1993).

Metabolic Alterations and Ca^{2+} Handling

Several metabolic changes which accompany fatigue might disrupt Ca^{2+} release and uptake by the SR as well. First, the accumulation of H^+ during fatigue reduces the pH resulting in depressed Ca^{2+} uptake and the amount bound to the SR (Fabiato and Fabiato, 1978) due to competition of H^+ and Ca^{2+} for binding sites on the ATPase, and diminished formation of the phosphorylated intermediates required for catalytic activity. Williams *et al.*, (1992) showed that the process of Ca^{2+} -induced Ca^{2+} release is also depressed by lowering pH. Second reduction of high energy phosphates (ATP and CP) and increase of the products of their degradation (ADP and Pi) affect the release and uptake of Ca^{2+} by the SR. Further, the loss of glycogen could alter SR function since the transverse tubule membrane bind the glycolytic enzymes aldolase and glyceraldehyde-3-phosphate dehydrogenase (Williams and Klug, 1995). The finding that incubation of the rat diaphragm in high levels of glucose-6-phosphate causes a slight increase in contractility supports this notion (Varagic and Zugic, 1971). Moreover, unsaturated free fatty acids (FFA's) facilitate the release of Ca^{2+} and reduce the threshold for Ca^{2+} induced Ca^{2+} release (Messineo *et al.*, 1984; Fletcher *et al.*, 1991). Since intramuscular triglyceride concentrations are reduced following prolonged exercise, it is possible that their facilitory affect on the SR is removed (Williams and Klug, 1995).

Free Radicals and Ca^{2+} Handling

Finally, an increase in free radical production results in increased glutathione oxidation, lipid peroxidation and reduced levels of intracellular antioxidants due to sustained exercise (Davies *et al.*, 1982; Khawli and Reid, 1994) and cause some alteration in skeletal muscle SR which facilitates fatigue. The specific mechanism of free radical induced SR dysfunction remains undefined, however, a possible explanation is that free radicals influence fatigue by modulating calcium flux across SR. Two possible sites of action are the histidyl residue associated with the ryanodine-sensitive calcium-channel protein, and the calcium dependent ATPase of the SR. Both these regulatory proteins of the SR can be modulated by sulfhydryl oxidation caused by free radicals triggering rapid calcium efflux from terminal cisternae and decreased calcium uptake (Khawli and Reid, 1994). This notion is supported by studies (Scherer and Deamer, 1986), showing that changes in SR function such as depressed Ca^{2+} uptake and ATPase activity can be attenuated by antioxidants that limit free radicals.

The proposed experiments are not designed to elucidate the specific mechanism responsible for the diaphragmatic fatigue. However, since fatigue play a role in the pathogenesis of respiratory failure and in exercise limitation, it becomes an objective with exceedingly important therapeutic implications to examine if the well documented cellular adaptations of the diaphragm to endurance exercise affect the fatigue rate of the muscle *in vitro*. A hypothesis that has not yet been tested.

Summary

The respiratory muscles are plastic and their adaptive strategies to endurance exercise are similar, but of smaller magnitude, to those of locomotor muscles. The reason(s) for this difference in the magnitude of the training induced adaptations between respiratory and locomotor muscles is not well understood; however, two possible explanations are: First, it may be that exercise training places a smaller metabolic load on the diaphragm. Second, the basal mitochondrial enzyme activity in the costal diaphragm is significantly higher prior to training. The training induced respiratory muscle cellular adaptations include: 1) increases in oxidative enzyme activity, 2) increases in antioxidant enzyme activity, 3) decreases in fiber CSA, and thus increases in capillary dencity, and 4) slow to fast shifts in MHC isoforms. How these adaptations affect the contractile properties and fatigue rate of the diaphragm remains unknown. The precise mechanisms that mediate diaphragmatic fatigue have yet to be identified, however, it is generally accepted that EC coupling within the muscle fiber plays a major role in diaphragmatic fatigue. Evidence suggests an alteration in SR function of fatigued muscle that results in a reduction in the intracellular levels of Ca^{2+} due to a diminished SR Ca^{2+} release and/or uptake.

CHAPTER 3
METHODS

Animals

This project was approved by the University of Florida Institutional Animal Care and Use Committee (# 4205) and followed the guidelines for animal use established by the American Physiological Society.

Female Sprague-Dawley rats (120 days old) were obtained for these experiments. An animal model was chosen because the invasive nature of the experiments precluded the use of human subjects. Further, the physiological function and fiber type distribution of the rat diaphragm resembles the human diaphragm, and thus it is an excellent model to study respiratory muscle function. The Sprague-Dawley rat was chosen because this strain has been well characterized with respect to locomotor and respiratory muscle properties, and pilot data has demonstrated that it is an acceptable model for respiratory muscle studies (Powers 1994a; Powers 1994b; Powers 1992b; Powers 1990b).

Experimental Design

To test the hypotheses listed earlier (INTRODUCTION), endurance trained and sedentary rats were examined for diaphragmatic *in vitro* contractile properties, fatigue rate, muscle phenotype, and muscle oxidative and antioxidant capacity. On two separate occasions, a total of sixty-four female Sprague-Dawley rats were housed in pairs and fed rat chow and water *ad libitum* while being maintained on a 12/12 hour light/dark photoperiod for ~7 days prior to beginning the experiments. During this 7 day period, animals were handled daily to reduce contact stress. The animals were then assigned to one of two experimental groups (n = 15 per group) based on their willingness to run on a motorized treadmill.

Experiment 1 (n = 34): specific force, twitch characteristics, force-frequency relationship, and 10 min. fatigue protocol.

Group 1) control (restricted to cage activity).

Group 2) trained (10 weeks, 5 days/week, 60 min/day, at ~ 70% of VO_{2max}, treadmill running).

Experiment 2 (n = 30): force-velocity relationship, and 60 min. fatigue protocol.

Group 1) control (restricted to cage activity).

Group 2) trained (10 weeks, 5 days/week, 60 min/day, at ~ 70% of VO_{2max}, treadmill running).

This selection procedure for training animals is justified given the fact that health status and muscle physiological properties do not differ between young adult rats with a willingness to run and those animals resistant to exercise (Bedford, *et al.*, 1979). Trained animals underwent a 10 week training program consisting of running on a motor driven treadmill 5 days per week for 60 minutes. This training protocol has been shown repeatedly to induce increases in diaphragmatic oxidative and antioxidant capacity in our laboratory. Control animals were placed on the motorized treadmills, while they were turned off, two times per week, for five to ten minutes, in order for both groups to be subjected to the same stress levels and thus eliminate that extraneous factor.

The decision to assign 15 animals per group was based on an estimated statistical power analysis using data from previous investigations in our laboratory.

Exercise Training Protocol

The exercise training group run on a motorized treadmill five days per week, for the first three weeks, and four days per week thereafter, for a total of 10 weeks. On day one, the animals began exercising at 25 m/min (5 min duration / 0% grade). The duration of exercise was increased by five minutes per day until the animals reached sixty minutes of exercise at the end of the third week. The duration of exercise remained at sixty minutes for the duration of the study. During the fourth week, the treadmill speed was increased by approximately 1 m/min per day until a speed of 30 m/min was reached. The speed remained constant through weeks six to ten. Beginning week four, the treadmill grade was increased by three percent every week until reaching a zenith of 18% grade during week nine of training. The training protocol is outlined in Table 1. The work loads depicted represent approximately 70% of the animals $VO2_{max}$ and the progression assumes a 15-20% improvement over the training period. The exercise training protocol selected for use in this study has been described previously and has been shown to significantly improve diaphragmatic oxidative and antioxidant capacity in young rats in our laboratory (Powers *et al.*, 1992b; Powers *et al.*, 1994a).

Table 1. Ten week motorized treadmill training protocol for trained female, Sprague-Dawley rats.

Week (#)	speed (m/min)	grade (%)	durarion/day (min)
1	25	0	20
2	25	0	40
3	25	0	60
4	30	3	60
5	30	6	60
6	30	9	60
7	30	12	60
8	30	15	60
9	30	18	60
10	30	18	60

The above work loads represent approximately 70% of the animals $VO2_{max}$; the progression assumes a 15-20% improvement over the training period.

Experimental Protocol

Animals were euthanized by intraperitoneal injection of sodium pentobarbital (90mg/kg) forty-eight hours after the last training session, and the entire diaphragm was quickly removed and placed in a dissecting dish. A small strip of the costal diaphragm (~0.3 x ~2.0 cm) was carefully cut leaving a portion of the central tendon and the rib attachment on the ends. This strip was utilized for *in vitro* measurements of contractile function; specifically, peak isometric twitch tension (P_t), one-half relaxation time (i.e. the time required for force to fall from maximum to half-maximum; (1/2 RT)), maximal rate of twitch tension development (dP/dT), peak isometric tetanic tension (P_o), isotonic force-velocity relationship, at 25° C, and force-frequency relationship, and fatigue rate, at 37° C. All contractile measurements were conducted with the muscle at optimal length (L_o). Following the measurements, the length of the muscle strip (L_o) and its mass were carefully determined for calculation of muscle cross-sectional area (CSA) using the formula:

$$\text{CSA (cm}^2\text{)} = \text{muscle mass (g)} / [\text{muscle length (cm)} \times \text{muscle density (g/cm}^3\text{)}]$$

Assuming muscle density = 1.056 g/cm^3 (Metzger and Fitts, 1986).

The remaining diaphragm was carefully trimmed of fat and connective tissue (including the central tendon). The costal portion of the diaphragm was then divided into two pieces: piece #1 was frozen in liquid nitrogen and stored at -70° C for subsequent measurement of myosin heavy chain (MHC) and myosin light chain (MLC) profile; piece #2 was frozen in liquid nitrogen for subsequent measurement of CS, total SOD, and GPX. The crural portion of the diaphragm was frozen in liquid nitrogen for subsequent measurement of CS, total SOD, and GPX.

In order to provide a locomotor muscle comparison of the effects of endurance training on the diaphragm, the right and left plantaris muscles were also removed, frozen in liquid nitrogen and stored at -70° C for measurement of CS.

Experimental Procedures

Diaphragm Strip *in Vitro* Contractile Measurements

After the animal reached a surgical plane of anesthesia, the entire diaphragm was removed and placed in a dissecting chamber containing a Krebs-Hensleit solution equilibrated with a 95% O_2 / 5% CO_2 gas. A muscle strip was dissected out from the ventral costal region and suspended vertically between two light weight Plexiglas clamps and connected to a transducer (Cambridge Technology, model 300B) in a jacketed tissue bath containing Krebs-Hensleit and 12μM d-tubocurarine to produce complete neuromuscular blockade. The Cambridge transducer is a combined force and position transducer and is capable of monitoring both isometric and isotonic contractions. The force on the lever can be electronically controlled from 0 to 80 g while the length change of the muscle is monitored, or the length of the muscle can be held constant while force is monitored. The jacketed tissue bath was aerated with gas (95% O_2 / 5% CO_2), pH was maintained at 7.4, and the osmolality of the bath was ~ 290 mOsmol. Temperature in the organ bath was maintained at 25 ±0.5° C for measurement of P_o, P_t, 1/2 RT, dP/dT, and the force-velocity relationship to minimize temperature dependent deterioration of the preparation. After 15 minutes of thermo-equilibration in the bath, the muscle strip was stimulated along its entire length with platinum wire electrodes using a modified Grass Instruments S48 stimulator. A supramaximal stimulation voltage was used equal to ~150% of the minimum voltage necessary for maximal activation of the muscle (140 volts; 2 msec duration). We have demonstrated in pilot experiments that this method of stimulation results in maximal force generation when compared to direct muscle stimulation using stainless steel electrodes.

After the 15 minute equilibration period, the muscle strip was adjusted to its optimum contractile length (L_O) at which maximal tetanic tension was obtained; this was accomplished by systematically adjusting the length of the muscle using a micrometer while evoking tetanic contractions (140 volts, 330 msec train duration, 100 Hz, and 2 msec pulse width).

After the collection of the twitch and tetanic parameters, temperature was increased and maintained at 37 ±0.5° C, by means of a temperature controller circulating water through the outer jacket of the bath. Temperature was monitored by a YSI tele-thermometer (model 2100, Yellow Springs Instruments, Yellow Springs, OH). Twenty-five minutes were allowed for the diaphragm strip to equilibrate at that temperature. Following the thermo-equilibration period measurements of the force-frequency relationship as well as the fatigue and recovery rates were conducted.

Isometric twitch contractions

Peak isometric twitch tension was determined from a series of single pulses (140 volts, 2 msec duration). The Cambridge transducer output was amplified and differentiated by operational amplifiers and underwent A/D conversion for analysis using a computer based data acquisition system (GW Instruments-Series

II). In addition, 1/2 RT, and dP/dT was determined by computer analysis of the force transduced output.

Peak isometric tetanic tension

A series of isometric tetanic contractions were produced using a supramaximal (140 V) stimulus train of 100 Hz, 2 msec pulse width, and 330 msec duration. Force was monitored by a computerized ergometer previously described. Each tetanic contraction was separated by a two minute recovery period.

Force-velocity measurements

The relationship between force and muscle shortening velocity was assessed by measurement of shortening velocity (Cambridge transducer model 300B) at ~12 isotonic loads (100 msec trains of 2 msec pulses at 100 Hz) over the range of ~2-90% of P_o at 25 ± 0.5° C. A two minute time period elapsed between contractions. The force-velocity data was fit to the Hill equation (Woledge *et al.*, 1985) using least-squares techniques, and maximal velocity of unloaded shortening (V_{max}) was determined by solving for velocity when force equals zero.

Force-frequency measurements

Response of the diaphragm muscle strip to increasing stimulus frequency was assessed at L_o at 37 ± 0.5° C, by the application of 15, 30, 40, 50, 80, 120, 160, 200 Hz pulses applied in 330ms trains. A two minute time period elapsed between contractions.

Fatigue and recovery measurements

In two separate experiments, two different fatigue protocols were used, and fatigue rate was measured at 37 ± 0.5° C by monitoring the decrease in the costal diaphragm strip force production, over time. Values were expressed as percent of the initial value at time zero. In both occasions the muscle was stimulated by isometric unfused tetanic contractions, using a supramaximal (140 V) stimulus train of 30 Hz, every 2 seconds with a train duration of 250 msec. The duty cycle used therefore, that is, the ratio of the period of muscle contraction (250ms) to the duration of a cycle of contraction and rest (2000ms), was 12.5%. In the first experiment, fatigue rate was measured over a period of ten minutes, were recovery rate was followed for five minutes. Since during *in vitro* stimulation all muscle fibers contract simultaneously, which is not the case *in vivo*, we postulated that the approximately 50% decrease in force production that was observed during the ten minutes of stimulation in this experiment, was due to fatigue of the fast fibers. The CSA of the diaphragm is consisted by approximately 60% of type IIb and IIdx fibers, where as, type I and IIa fibers comprise 40% of the diaphragm. In the second experiment therefore, the fatigue protocol was extended to sixty minutes, in order to compare the fatigue pattern of the type I and IIa fibers that were predominantly affected from endurance exercise. The results supported our postulate. For the rational of the stimulation paradigms used, see DISCUSSION.

Biochemical Measurements

Isolation of myofibrillar protein

Myofibrillar protein was isolated using a modification of the myofibril extraction technique described by Solaro *et al.* (1971); myofibrillar protein concentration was then determined using the biuret technique of Watters (1978). Briefly, the costal diaphragm and plantaris portions designated for myofibrillar protein isolation were thawed and placed in a petri dish on ice. The muscle was then carefully cleaned of fat and tendon and scissor minced. Precisely 100 mg of muscle was then homogenized in 2 ml of sucrose buffer and centrifuged for 10 min. at 1000 x g. The supernatant was discarded and the pellet suspended in a KCl buffer containing Triton X-100 to eliminate membrane ATPase components. The pellet was again homogenized and centrifuged at 1000 x g. This process was repeated in a KCl buffer yielding a myofibrillar protein pellet of sufficient purity for quantitative assessment of myofibrillar concentration (Caiozzo *et al.*, 1992). For more details, see APPENDIX B.

Myosin heavy chain composition

The remaining myofibrillar protein (after biuret assay) was used for separation of MHC isoforms using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The myofibrillar protein was diluted in glycerol to a concentration of ~1 mg/ml (the samples can be stored in this form at -20° C for up to two months). These glycerol samples were further diluted to a concentration of ~0.25 mg/ml in sample buffer containing 62.5 mM Tris (pH=6.8), 1.0% SDS, 0.01% bromophenol blue, 15.0% glycerol, and 5.0% ß-mercaptoethanol. The protein was then denatured by incubation at ~95° C for 4 min.

One to three µg of protein was loaded onto 22 cm vertical gels (Biorad^{TM} Protean IIxi) composed of 8% acrylamide and 40% glycerol. The samples were electrophoresed for 22 hours at ~5° C using a constant voltage required to attain an initial current of 12 milliamps per gel (Talmadge and Roy, 1993). Following electrophoresis, the gels were stained with Coomassie Blue R-250, and destained in 30% methanol / 7% glacial acetic acid solution by diffusion.

Myosin light chain composition

The remaining myofibrillar protein was used for separation of MLC isoforms using SDS-PAGE. The myofibrillar homogenate was diluted in sample buffer and was loaded onto 6-cm long vertical gels and electrophoresed for 1 hour at ~24° C using SDS-PAGE (4-20% gradient gel). Gels were stained with Coomassie Blue R-250 and destained with a 30% / 7% methanol and glacial acetic acid solution. For more details, see APPENDIX C.

Quantification of myosin heavy and light chains

The relative proportions of the myosin isoform bands analyzed using a computer-based densiometric image analysis system including a Targa M8 image capture board (Truevision), and Java video analysis software (Jandel Scientific) integrated with a high resolution CCT video camera (Video World, Inc.). Each protein band was digitized in duplicate and the average was used for analysis. The coefficient of variation for repeated measurement of relative MHC and MLC composition of a given sample is<3.0%.

Tissue homogenization for enzyme assays

Homogenization of the tissue included 10 passes of a mechanically-rotated conical glass pestle through the homogenate in a tight-fitting 15ml glass homogenization tube (Kontes Tissue Grinder Size 23, Vinelant, NJ). We have demonstrated previously that this homogenization technique is proficient in cellular and mitochondrial disruption in the rat diaphragm while not introducing the damage that could be created by excessive passes (Fletcher *et al.*, 1995). Homogenates were then centrifuged (3° C) for 10 min at 400 x g. The resulting supernatant was decanted and assayed for CS, total SOD, and GPX. All tissue samples were homogenized in cold 100 mM phosphate buffer with 0.05% bovine serum albumin (1:20 wt/vol; pH = 7.4).

Enzyme assays

CS activity was determined using the procedures described by Srere (1969). Total SOD activity was determined using an improved spectrophotometric assay based on epinephrine autoxidation, described by Sun and Zigman (1978). GPX activity was determined using the procedures described by Flohe and Gunzler (1984). For details of these procedures, see APPENDIX A. In our laboratory the coefficients of variation for CS, SOD, and GPX assays are approximately 5, 4, and 5%, respectively. All enzyme assays were performed in duplicate at 25° C and samples from the control and experimental groups were assayed on the same day to reduce interassay variation.

Statistical Analysis

Comparisons between experimental groups (trained vs. untrained) for each dependent variable were made by unpaired t-test. Fatigue and force-frequency characteristics were evaluated for differences among groups with Student's t test for unpaired data corrected for multiple comparisons according to Bonferroni (0.05 / # of comparisons = α). Significance was established at $P<0.05$. Data are expressed as means ± SEM.

CHAPTER 4
RESULTS

Morphometric Characteristics

Mean (±SEM) body mass and morphometric characteristics of the diaphragm for both experimental groups are presented in table 2. Body mass did not differ between groups before or after the completion of the ten week training protocol. Likewise, the dimensions of the costal diaphragm *in vitro* strips (length and CSA) taken at L_o were not different between groups.

Contractile Properties of the Costal Diaphragm

Table 3 reports the *in vitro* contractile properties (means±SEM) of costal diaphragm strips taken from control and trained animals. Maximal tetanic force, as well as, maximal twitch force expressed in ($N \bullet cm^{-2}$) did not differ between the two experimental groups (P_o: 24.32±0.5 vs 25.52±0.4; P_t: 9.04±0.45 vs 9.74±0.35). Therefore, the twitch to tetanus ratio was not different between control and trained costal diaphragm strips (0.374±0.021 vs 0.382±0.013).

In vitro twitch characteristics at 25^o C (1/2 RT and dP/dT) did not differ between the two experimental groups. Analysis of the isotonic force-velocity relationship fitted with the Hill equation (Woledge *et al.*, 1985) indicated no differences in V_{max} between control and trained diaphragm strips (6.57 ± 0.20 vs 6.35 ± 0.13 lengths • sec^{-1}). The a/P_o ratio did not differ between control and trained groups indicating similar curvature in the force-velocity relationship of the two experimental groups (0.19±0.009 vs 0.19±0.005). The Hill equation was fitted to the force-velocity data from each *in vitro* strip individually to calculate V_{max} and the a/P_0 ratio. However, for illustrative purposes, figure 3 presents the Hill equation fitted to the combined data for each group.

The force-frequency curves at 37° C for the control and trained groups are shown in figure 4. Mean (±SEM) data for production of costal diaphragm strip specific force ($N \bullet cm^{-2}$) were plotted against stimulation frequencies ranging from 15 to 200 Hz. Specific force was not different between control and trained costal strips at all stimulation frequencies and reached a plateau at 160 Hz in both groups.

Fatigue Rate of the Costal Diaphragm

Table 4 presents the *in vitro* ten minute fatigue and five minute recovery rate protocol at 37°C. No difference ($p<0.05$) existed in the rate of decline of force over time, expressed as a percent of the initial force produced at time zero, between control (48.5±1.9) and trained (53.5±2.1) costal diaphragm strips. Further, at one (63.6±3.2 vs. 64.6±2.8), three (63.6±2.9 vs. 63.9±2.8), and five (64.7±3.0 vs. 63.2±2.8) minutes of recovery, force production between groups was not different. These fatigue and recovery patterns are illustrated in figure 1.

Table 5 presents the sixty minute fatigue rate protocol at 37° C that was conducted at a separate occasion. Notice that the rate of fatigue statistically differentiated ($p<0.01$ according to Bonferroni corection for multiple comparisons) between the two groups after fifteen minutes into the fatigue protocol, and was different thereafter (30, 40, and 50 min). During the last thirty minutes of the fatigue protocol, the control costal diaphragm strips produced significantly less force (~26%), expressed as a percent of the initial force produced at time zero, compared to the trained diaphragms (~31%). The rate of force decline for both groups is illustrated in figure 2.

Oxidative and Antioxidant Enzymes

CS, SOD and GPX activities (means±SEM) are presented in table 6 for the costal and crural diaphragm and plantaris muscles of control and trained rats. Costal diaphragm CS ($\mu M \bullet min^{-1} \bullet 100mg\ prot.^{-1}$) and SOD ($units / min^{-1} \bullet 100mg\ prot.^{-1}$) activities were higher ($P<0.05$) in the trained group compared to the control (Figures 5 and 6). Mean (±SEM) values (control vs. trained) were as follows: CS: 54.2±1.3 vs. 59.7±1.0 ($P=0.001$), SOD: 3354.3±151.4 vs. 3763.1±122.1 ($P=0.02$). Further plantaris CS activity was 44% higher ($P<0.05$) in trained (39.9±1.2) compared with control (27.7±2.1) animals (Figure 5). There was no difference in the GPX activity ($\mu M \bullet min^{-1} \bullet 100mg\ prot.^{-1}$) of the costal diaphragm between the two groups (34.9±1.4 vs. 33.5±0.9) (Figure 7). In addition, no differences were observed for any of the enzymes measured for the crural diaphragm (CS: 51.8±1.6 vs. 52.2±0.9; GPX: 33.5±1.1 vs. 31.5±1.3; SOD: 3370.3±166.7 vs. 3396.8±128.4) (Figures 5, 6, and 7).

Myosin Heavy and Light Chain Isoforms

The relative myosin heavy chain isoform composition (mean±SEM) of the costal diaphragm for the control and trained animals is presented in table 7. A significant decrease ($p< 0.05$) in type IIb MHC (trained vs. control) (7.2±1.5 vs 10.9±1.0%) and an increase ($p< 0.05$) in type I MHC (36.1±0.7 vs 32.7±0.8%) was observed in the trained diaphragms, compared with control. Figure 9 shows a sodium dodecyl sulfate-polyacrilamide gel electrophoresis separation of the MHC isoforms. A comparison of the costal diaphragm MHC isoform profiles between groups is illustrated in figure 8. A significant reduction in type IIb MHC isoform content can be seen in the trained group, while type I MHC distribution was significantly increased in control animals.

Table 8 presents the relative MLC isoform composition (mean±SEM) of the costal diaphragm. No alterations were observed in the relative proportions of any of the types of MLC between groups. Finally, figure 10 illustrates an SDS-PAGE separation of the MLC isoforms of the costal diaphragm.

Table 2. Experiment 1. Morphometric characteristics of control and trained female, Sprague-Dawley rats.

	Control (n=14)	Trained (n=15)	P-value
Initial body mass (g)	243.9 ± 3.1	243.4 ± 4.3	NS
Final body mass (g)	276.3 ± 4.6	287.6 ± 6.1	NS
L_o (mm)	21.8 ± 0.5	22.4 ± 0.2	NS
CSA (cm^2)	0.01676 ± 0.00059	0.01809 ± 0.00085	NS

Values are means ± SEM.
NS = non-significant ($P>0.05$).
L_o = *In vitro* costal strip optimal length.
CSA = *In vitro* costal strip cross sectional area.

Table 3. Experiment 1. *In vitro* contractile properties of costal diaphragm strips from control and trained female, Sprague-Dawley rats.

	Control (n=12)	Trained (n=17)	P-value
Maximal tetanic P_o ($N \cdot cm^{-2}$)	24.32 ± 0.57	25.52 ± 0.45	NS
Maximal twitch P_t ($N \cdot cm^{-2}$)	9.04 ± 0.45	9.74 ± 0.35	NS
P_t / P_o ratio ($N \cdot cm^{-2}$)	0.374 ± 0.021	0.382 ± 0.013	NS
1/2 RT (msec)	48.71 ± 1.9	49.62 ± 2.1	NS
+dP/dt ($N \cdot msec^{-1} \cdot cm^{-2}$)	0.24 ± 0.01	0.26 ± 0.01	NS
* V_{max} ($lengths \cdot sec^{-1}$)	6.57 ± 0.20	6.35 ± 0.13	NS
a/P_o	0.19 ± 0.009	0.19 ± 0.005	NS

Values are means ± SEM.
NS = non-significant ($P>0.05$).
P_o = Isometric tetanic specific force.
P_t = Isometric twitch specific force.
1/2 RT = Relaxation half-time following a twitch contraction.
+dP/dt = Maximal rate of specific force development during a twitch contraction.
V_{max} = Maximal shortening velocity calculated from the Hill equation.
a/P_o = Curvature of the force-velocity relationship, where a = a constant derived from the Hill equation.
* = Experiment 2.

Table 4. Experiment 1. *In vitro* ten minute fatigue and five minute recovery rate (percent of initial force production) of costal diaphragm strips from control and trained female, Sprague-Dawley rats.

Time (minutes)	Control (n=13)	Trained (n=15)	P-value
Fatigue			
1	106.1 ± 2.7	105.7 ± 4.9	NS
4	67.5 ± 2.8	68.6 ± 3.1	NS
6	59.4 ± 2.5	60.0 ± 2.6	NS
8	53.2 ± 2.7	54.9 ± 2.7	NS
10	48.5 ± 1.9	53.5 ± 2.1	NS
Recovery			
11	63.6 ± 3.2	64.6 ± 2.8	NS
13	63.6 ± 2.9	63.9 ± 2.6	NS
15	64.7 ± 3.0	63.2 ± 2.8	NS

Values are means ± SEM.
NS = non-significant (P>0.05).

Table 5. Experiment 2. *In vitro* sixty minute fatigue rate (percent of initial force production) of costal diaphragm strips from control and trained female, Sprague-Dawley rats.

Time (minutes)	Control (n=13)	Trained (n=15)	P-value
16	31.9 ± 1.9	41.4 ± 2.2	P=0.002
30	25.2 ± 1.4	31.6 ± 2.1	P=0.01
40	26.6 ± 1.3	31.1 ± 1.5	P=0.01
50	24.8 ± 1.3	31.4 ± 2.1	P=0.007
60	27.7 ± 1.0	31.5 ± 1.0	NS (P=0.03)

Values are means ± SEM.
NS = non-significant (P>0.01 according to Bonferroni correction).

Table 6. Experiment 1. Citrate synthase (CS), glutathione peroxidase (GPX), and superoxide dismutase (SOD) activities of costal and crural diaphragm and plantaris from control and trained female, Sprague-Dawly rats.

	Control (n=14)	Trained (n=18)	P-value
Costal Diaphragm			
CS	54.2 ± 1.3	59.7 ± 1.0	P=0.001
GPX	34.9 ± 1.4	33.5 ± 0.9	NS
SOD	3354.3 ± 151.4	3763.1 ± 122.1	P=0.02
Crural Diaphragm			
CS	51.8 ± 1.6	52.2 ± 0.9	NS
GPX	33.5 ± 1.1	31.5 ± 1.3	NS
SOD	3370.3 ± 166.7	3396.8 ± 128.4	NS
Plantaris			
CS	27.7 ± 2.1	39.9 ± 1.2	P=0.0001

Values are means ± SEM.
NS = non-significant ($P>0.05$).

Units for CS and GPX ($\mu M \cdot min^{-1} \cdot 100mg\ prot.^{-1}$) = Micromoles of substrate converted per minute per 100 milligrams of protein.

Units for SOD ($units \cdot min^{-1} \cdot 100mg\ prot.^{-1}$) = One unit is equal to the amount of activity which causes 50% inhibition of Epinephrine oxidation.

Table 7. Experiment 2. Myosin heavy chain (MHC) isoform composition (percent of total MHC pool) of costal diaphragm from control and trained female, Sprague-Dawley rats.

	Control (n=13)	Trained (n=14)	P-value
Type I MHC	32.7 ± 0.8	36.1 ± 0.7	P=0.004
Type IIa MHC	20.7 ± 0.9	22.0 ± 1.7	NS
Type IIdx MHC	35.6 ± 0.5	34.5 ± 1.0	NS
Type IIb MHC	10.9 ± 1.0	7.2 ± 1.5	P=0.03

Values are means ± SEM.
NS = non-significant (P>0.05).

Table 8. Experiment 2. Myosin light chain (MLC) isoform composition (percent of total MLC pool) of costal diaphragm from control and trained female, Sprague-Dawley rats.

	Control (n=14)	Trained (n=15)	P-value
MLC 1s	9.3 ± 1.1	8.5 ± 0.7	NS
MLC 1f	24.6 ± 1.0	23.9 ± 2.0	NS
MLC 2s	5.5 ± 1.0	6.3 ± 1.3	NS
MLC 2f	48.1 ± 2.1	49.4 ± 1.6	NS
MLC 3f	12.2 ± 1.1	11.5 ± 1.1	NS

Values are means ± SEM.
NS = non-significant (P>0.05).

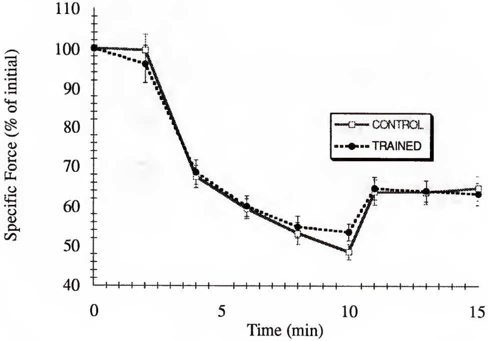

Figure 1. Experiment 1. *In vitro* ten minute fatigue and five minute recovery rates of costal diaphragm strips from control and trained animals. Values are means ± SEM. No differences were observed between groups.

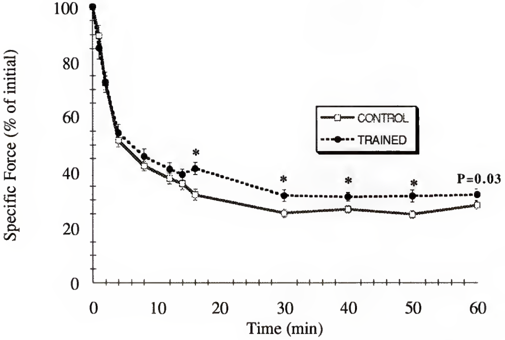

Figure 2. Experiment 2. *In vitro* sixty minute costal diaphragm strip fatigue rate for control and trained animals. Values are means ± SEM. * Different from control ($P<0.01$ according to Bonferroni corection for multiple comparisons).

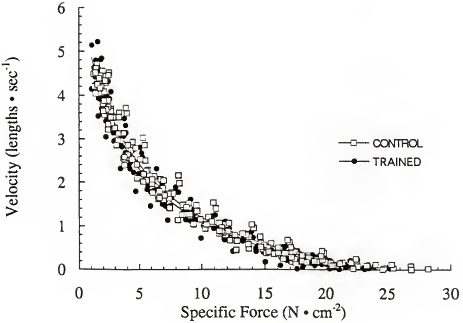

Figure 3. Experiment 2. Force-velocity relationships for costal diaphragm *in vitro* strips from control and trained female, Sprague-Dawley rats. The Hill equation was fitted to the force-velocity data from each *in vitro* strip individually to calculate V_{max} and the a/P_0 ratio. However, for illustrative purposes, figure 3 presents the Hill equation fitted to the combined data for each group.

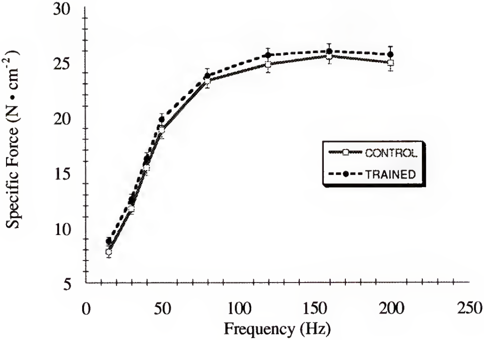

Figure 4. Experiment 1. Force-frequency relationship of *in vitro* costal diaphragm strips from control and trained female, Sprague-Dawley rats. Values are means ± SEM.

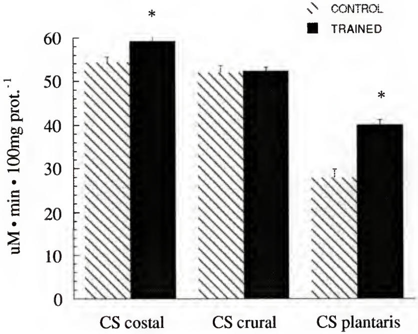

Figure 5. Comparison of costal and crural diaphragm and plantaris CS activity between control and trained animals. Values are means ± SEM. * Different from control ($P<0.05$).

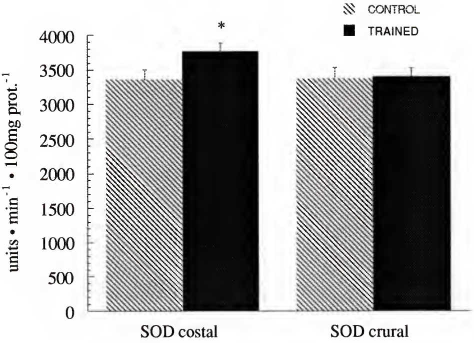

Figure 6. Comparison of costal and crural diaphragm SOD activity between control and trained animals. Values are means ± SEM. * Different from control ($P<0.05$).

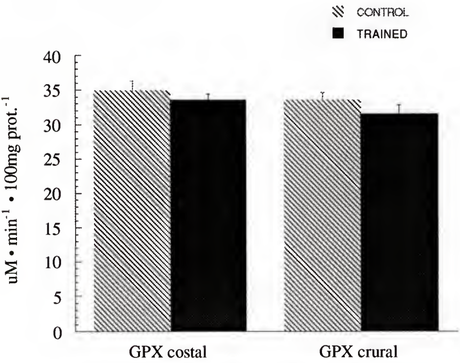

Figure 7. Comparison of costal and crural diaphragm GPX activity between control and trained animals. Values are means ± SEM.

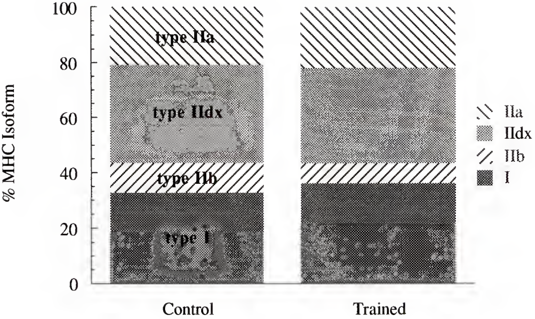

Figure 8. Comparison of costal diaphragm myosin heavy chain isoform profiles between control and trained animals. Note the significant reduction in type IIb MHC isoform content, while type I MHC distribution is significantly increased.

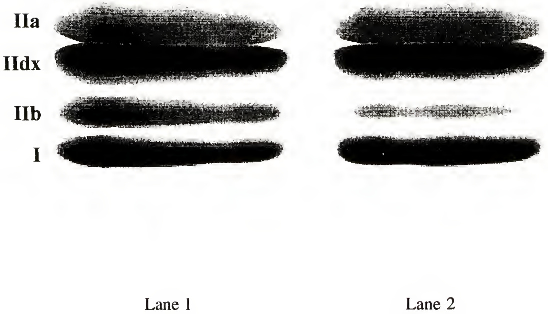

Figure 9. Photograph of a typical sodium dodecyl sulfate-polyacrylamide gel electrophoresis of bundles of fibers from the costal diaphragm illustrating the region of the gel containing the myosin heavy chains. Lane 1 contains a control sample; lane 2 contains a trained sample.

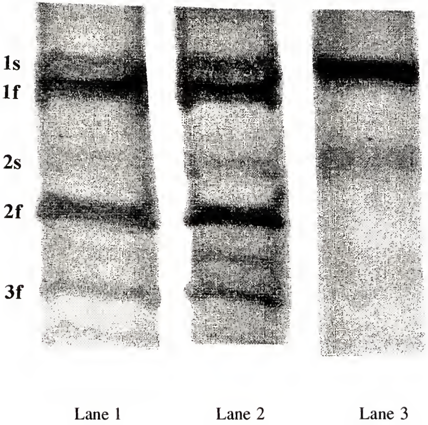

Figure 10. Photograph of a typical sodium dodecyl sulfate-polyacrylamide gel electrophoresis of bundles of fibers from the costal diaphragm illustrating the region of the gel containing the myosin light chains. Lane 1 contains a control costal diaphragm sample; lane 2 contains a trained costal diaphragm sample; lane 3 contains a soleus sample.

CHAPTER 5
DISCUSSION, SUMMARY, CONCLUSIONS, IMPLICATIONS FOR FUTURE RESEARCH

Overview and Principle Findings

This study tested three hypotheses: 1) Endurance training will reduce the rate of diaphragmatic fatigue development *in vitro*. 2) Endurance training will result in decreased diaphragmatic maximal shortening velocity (V_{max}) and therefore decreased maximal power output. 3) Endurance training will not alter diaphragmatic specific force.

The data support hypothesis #1 that endurance training reduces the rate of diaphragmatic fatigue *in vitro*. This conclusion is corroborated by data that indicates significant increases in the activities of the tricarboxylic acid cycle marker enzyme CS and the antioxidant enzyme SOD. Further, hypothesis #2 is not supported by the data which showed no difference in trained diaphragmatic V_{max} compared to controls despite the decrease in type IIb MHC and the small increase in type I MHC in trained diaphragms. Finally, in support of hypothesis #3, endurance training did not affect diaphragmatic specific force.

In Vitro Fatigue Rate in Costal Diaphragm Strips

This study is the first to document an endurance training -induced reduction in the decline of the force output, that is observed in the fatiguing costal diaphragm *in vitro*. Our results are consistent with investigators who have observed improvement in respiratory muscle endurance in healthy subjects and patients with airway disease as a result of respiratory muscle training (Leith and Bradley, 1976; Aldrick and Karpel, 1985; Clanton *et al.*, 1985; Morgan *et al.*, 1987; Belman and Gaesser, 1988; Fairbarn *et al.*, 1991; Levine *et al.*, 1986; O'Kroy and Coast, 1993). The results of this report, are not in agreement with the study of Metzger and Fitts (1986) that examined the effects of endurance training on the fatigueability of the rat diaphragm. These authors reported that training had no effect on the fatigue rate of the costal diaphragm. However, the training stimulus that was used in their study was insufficient to induce increases in the oxidative capacity of the diaphragm. It is not surprising therefore, that the comparison of diaphragms with no biochemical, morphological or phenotypic difference would not reveal contractile differences. Moreover, the stimulation paradigm that was used in their study, was of high-frequency (50 Hz; P_0 at 22°C = 50 Hz) and short duration (10 min). The fatigue data from our study argues against the use of both high-frequency and short duration stimulation protocols *in vitro*.

The uniqueness of our approach to the problem is discussed in the next several paragraphs.

Trained vs. Untrained Costal Diaphragm Strips *in Vitro*

We used a training protocol that has been shown to improve oxidative and antioxidant capacity of the rat costal diaphragm as well as, to reduce the costal diaphragmatic fibers CSA (i.e. Powers *et al.*, 1992a; Powers *et al.*, 1994a). The oxidative and antioxidant enzyme activity data, indicates that we were successful in our approach. Indeed, CS and SOD activities were 10.1% and 12.1% respectively higher ($p < 0.05$) in the trained diaphragms compared with the controls. Further, type I MHC in the trained diaphragms was 10.4% higher ($p< 0.05$) than controls, and type IIb was 33.9% lower, revealing a fast to slow shift. The present investigation is, therefore, the first to examine the effects of endurance exercise on the fatigue rate of the diaphragm comparing trained and control costal diaphragm strips.

In Vitro Bath Temperature

The present investigation studied diaphragmatic fatigue *in vitro* at 37° C. Diaz and Clanton (1994), studied the effects of the antioxidant N-acetylcysteine on *in vitro* diaphragmatic fatigue. Experiments performed at 26° C showed minor effects of this antioxidant with regard to its fatigue sparing properties. Experiments performed at 37° C demonstrated large effects of N-acetylcysteine on *in vitro* diaphragmatic fatigue. These results clearly demonstrated that the effects of this antioxidant on *in vitro* diaphragm fatigue are temperature dependent. Although the mechanism for this thermal effect is not clear, Diaz and Clanton (1994) hypothesized that repetitive unfused contractions at 37° C *in vitro* result in more metabolic activity, compared with lower temperatures and thus greater free radical production. Based on this finding, to test the hypothesis that the exercise induced increase in SOD affects fatiguability of the diaphragm strips *in vitro*, the temperature of the bath was 37° C, comparable to that used by others (Reid and Miller, 1989; Khawli and Reid, 1994; Reid *et al.*, 1992a; Shindoh *et al.*, 1990; Reid *et al.*, 1992b; Supinski *et al.*, 1991; Diaz and Clanton 1994).

Metzger and Fitts (1986), conducted their experiments at 22 °C and argued that higher temperatures deteriorate the preparation. Pilot experiments from our laboratory revealed that the preparation is viable for several hours at 37°C depending on the frequency of stimulation (unpublished observations).

Results from Khawli and Reid, (1994) who studied the effects of 37° C over a two hour period on the viability of similar size diaphragm strips *in vitro* support our findings demonstrating that the relative stability of the preparation mimic the stability of isolated limb muscle at cooler bath temperatures of 20-30°C. They attributed the stability of the preparation to infrequent stimulation, and to the thinness of the diaphragm strips, since diffusion distance for oxygen averaged 0.35 mm, well below the critical diffusion distance of ~0.58 mm reported by Segal and Faulkner (1985) for resting muscle at 37 °C.

Duration of the *in Vitro* Stimulation Paradigm

Two separate fatigue experiments were conducted in this study. Fatigue was induced using the same low frequency, small duty cycle stimulation paradigm in both experiments. Our initial studies performed with a common used fatigue protocol duration of ten minutes, and showed no effects of the treatment in the rate of fatigue, between control and trained costal diaphragms. The mean percent decrease in the force production after ten minutes of stimulation in this experiment was 48.5±1.9% for the control and 53.5±2.1% for the trained diaphragm strips. Due to the trend of the trained diaphragm strips to produce higher forces at the tenth minute of stimulation, we postulated that the approximately 50% decrease in force production, represents fatigue of the type IIb and IIdx fibers which consists approximately 60% of the CSA of the diaphragm and is masking the fatigue pattern of the type I and IIa fibers that comprise 40% of the diaphragm. Our repeat experiments at a longer duration fatigue protocol were conducted, to discern differences in the fatiguability of the type I and IIa fibers, that are predominantly contributing to the chronic force production of the diaphragm, expanding the chest wall. The repeat experiments demonstrated significant effects of the treatment; effects that we believe are consistent with our postulate.

An important determinant of the use of the *in vitro* preparation as a model for evaluating contractile properties of diaphragm fiber bundles, is that the stimulus used to excite the muscle studied, is supramaximal. This ensures that all the muscle fibers are recruited, if valid comparisons are to be made between different treatments. When therefore the rate of fatigue is measured, fast fatiguable fibers are responsible for the initial decrease in force production where fatigue resistant fibers play a later role. Our hypothesis, was partially based on earlier findings demonstrating that addition of exogenous antioxidants and particularlly SOD prevented *in vitro* fatigue, acting directly on muscle myocytes (Reid *et al.*, 1992a). The significant differences in the rate of fatigue observed in that study using an identical stimulation protocol of ten minute duration, was due to the fact that the antioxidants added to the bath acted on all types of fibers including IIb. Our treatment (endurance exercise), however, affected predominantly the type I and IIa muscle fibers. Although, there is some controversy (Gosselin *et al.*, 1992a; Green *et al.*, 1989), Powers *et al.*, (1992a; 1992b), using histochemical staining for SDH activity, reported that the increase in the oxidative capacity of the diaphragm due to an exercise protocol identical with ours, was limited to type I and IIa muscle fibers. Demonstrating therefore, that these are the fibers mainly recruited during exercise. Sieck and Fournier (1989) have suggested that recruitment of diaphragmatic type IIb fibers may not be suitable for generating ventilatory forces and that may be used only in non ventilatory behaviors such as sneezing and gagging. This is a logical hypothesis since prevention of recruitment of the type IIb fibers during heavy ventilatory effort would act as a protective mechanism from fatigue in this vital organ.

It is therefore difficult to study the effects of training induced increase in oxidative and antioxidant capacity on the fatigue rate of the diaphragm, by using a short duration fatigue protocol, since *in vitro* all muscle fibers are maximally recruited simultaneously, which is not the case *in vivo*.

By extending our fatigue protocol beyond the point of ten minutes, we compared the fatiguability of these fibers (I and IIa) that were adapted to the stress of exercise. The results of the sixty minute fatigue protocol demonstrate that the magnitude of the percent force reduction, between control and trained diaphragm strips, statistically differentiated ($p<0.05$) after fifteen minutes of stimulation and remained different until the termination of the experiment. This finding supports the aforementioned postulate.

Duty Cycle and Frequency of the *in Vitro* Electrical Stimulation Paradigm

The rationale for using a low duty cycle (12.5%), that is, the ratio of the period of muscle contraction (250 ms) to the duration of a cycle of contraction and rest (2000 ms), was based on findings from studies reporting that increases in duty cycle accelerate fatigue (Kelsen and Nochomovitz, 1982; Seow and Stephens, 1988). Kelsen and Nochomovitz (1982), demonstrated that the duty cycle per se appears to be the factor that determine the rate of fatigue, rather than the absolute duration of contraction and rest. The rate of fatigue in their study was the same at a given duty cycle, even though, the absolute lengths of the period of diaphragmatic contraction and the duration of a cycle of contraction and rest were varied over a wide range.

A pulse frequency of 30 Hz was chosen because it approximates the firing frequency of phrenic motoneurons during heavy ventilatory efforts since 15Hz approximates the firing frequency under eucapnic conditions (Iscoe *et al.*, 1976).

The unfused tetani, low-frequency (30 Hz; P_o at 37°C = 200 Hz) stimulation paradigm that was used in this study mimics the metabolic changes produced by peripheral fatigue *in vivo*. This type of protocol causes depletion of the phosphocreatine levels and glycogen stores, where decreases intracellular pH and increases Pi levels. Therefore fatigue develops due to inhibition of the actin and myosin interaction (Reid *et al.*, 1992a). Reid *et al.*, (1992a) demonstrated that diaphragm fiber bundles *in vitro* produce reactive oxygen intermediates, including $O^{-2\cdot}$ and H_2O_2 which promote low frequency fatigue in this preparation. In their study, addition of exogenous antioxidants inhibited low-frequency fatigue where development of high-frequency fatigue was unaffected. High frequency fatigue of curarized muscle is attributed to failure of impulse propagation across the sarcolema (Reid *et al.*, 1992a). This mechanism of fatigue is doubtful to occur during *in vivo* spontaneous contractions.

Isometric vs. Isotonic Fatigue *in Vitro*

Our results also argue against the use of *in vitro* isotonic rapid fatigue protocols when examining the effects of exercise on the diaphragm. Normal diaphragmatic function involves muscle shortening and therefore, *in vitro* isotonic measurements are more similar to *in vivo* diaphragmatic contractions. We used isometric contractions in these experiments for the following reasons: Experiments from our laboratory demonstrated that *in vitro* isotonic fatigue develops rapidly (Dodd *et al.*, 1995). Several other investigators showed that shortening of the muscle influences the development of fatigue, with muscle fatigue developing faster in isotonic contractions than in isometric ones (Mardini and McCarter, 1987; Seow and Stephens, 1988; Ameredes and Clanton 1990). Ameredes and Clanton (1990), postulated three possible mechanisms of how shortening can influence the development of fatigue. First, shortening may impose additional energetic cost, due to the energy required to detach and reattach cross bridges as the muscle shortens. Seow and Stephens (1988), also speculated that *in vitro* mouse diaphragm shortening resulted in an increased energy demand. Second, additional energy metabolism is required within muscles that performe external work, which may lead to less energy available for force development (Fenn effect). Ameredes and Clanton (1990), calculated that shortening contractions performed 84% greater work by CSA, compared with the average total work of isometric contractions of *in situ* diaphragm muscle strips. Third, shortening may impede blood flow, which could lead to pH -induced fatigue (Williams and Klug, 1995). This last mechanism does not apply to the non-perfused diaphragm *in vitro*.

Thus, despite the fact that isotonic contractions mimic diaphragmatic muscle shortening *in vivo*, muscle shortening induced rapid fatigue *in vitro*, does not mimic long-term, low frequency diaphragmatic fatigue *in vivo*. The use, therefore, of an isotonic fatigue protocol, would prohibit us from answering the major question of this study. The use of isometric fatigue enables us to mimic a more physiologic type of long duration, low-frequency fatigue which allows us to examine the effects of exercise on the fatiguability of the type I and IIa diaphragmatic fibers.

Based on the ideas discussed above, an *in vitro*, long-duration, low frequency, low duty cycle, isometric stimulation protocol was the only means of testing our experimental hypothesis.

Advantages of the *in Vitro* Model

Despite the limitations (see above) of the *in vitro* preparation, several advantages exist over studies performed *in vivo* (Kelsen and Nochomovitz, 1982).

First, the isometric forces developed by the costal diaphragm strip *in vitro* can be measured directly and not from inferences after measuring mouth or transdiaphragmatic pressure changes, using esophageal and gastric balloons. Transdiaphragmatic pressure changes can be influenced by changes in lung volume and thus diaphragmatic muscle length can not be controlled (Braun *et al.*, 1982). Therefore, interpretation of the results from studies performed *in vivo* requires grate caution since the muscle length-tension relationship can have major impact on the results. Pressure changes can also be influenced by thoracic configuration (Grassino *et al.*, 1978).

Second, the electrical stimulus delivered to the muscle *in vitro* can be controlled, which is impossible when phrenic nerve activity is determined reflexly or volitionally.

Third, the use of curarized muscle *in vitro* allows for assesment of the direct effects of the treatment to the bundle of fibers tested, with no neural and/or junctional influences.

Cellular Mechanisms for the Training Induced Fatigue Sparing Effect

Although these data do not provide a definitive answer as to the specific mechanism(s) responsible for the training induced fatigue sparing effect on the costal diaphragm, several potential explanations exist.

First, the finding that oxidative enzyme activity was increased in the costal diaphragm of our animals after ten weeks of endurance training is in agreement with previous reports (Grinton *et al.*, 1992; Lawler *et al.*, 1993; Powers *et al.*, 1992b; Powers *et al.*, 1994a; Powers *et al.*, 1992c; Powers *et al.*, 1992e; Powers *et al.*, 1990b; Sugiura *et al.*, 1992a; Sugiura *et al.*, 1990; Uribe *et al.*, 1992; Gosselin *et al.*, 1992). This increase in the oxidative capacity of the diaphragm, could: a) enhance fat utilization, b) reduce the rate of glycogen degradation and c) decrease the rate of lactate production (Gollnick *et al.*, 1985; Holloszy, 1967; Holloszy and Coyle 1984).

Enhancement of fat utilization increases the availability of unsaturated free fatty acids (FFA's) which facilitate the release of Ca^{2+} and reduces the threshold for Ca^{2+} induced Ca^{2+} release (Messineo *et al.*, 1984; Fletcher *et al.*, 1991).

Reduction of the rate of glycogen degradation, prevents glycogen depletion in the muscle and therefore, prevents alteration in SR function since the transverse tubule membrane bind the glycolytic enzymes aldolase and glyceraldehyde-3-phosphate dehydrogenase (Williams and Klug, 1995). The finding that incubation of the rat diaphragm in high levels of glucose-6-phosphate causes a slight increase in contractility supports this notion (Varagic and Zugic, 1971).

Reduction in the rate of lactate production, prevents the accumulation of H^+ during fatigue. This prevents the reduction of the pH which results in depressed Ca^{2+} uptake and the amount bound to the SR (Fabiato and Fabiato, 1978) due to competition of H+ and Ca^{2+} for binding sites on the ATPase. Furthermore, a prevention of pH decrease, inhibits the depression of the Ca^{2+} -induced Ca^{2+} release process (Williams *et al.*, 1992). These tissue changes may delay fatigue during periods of high intensity contractile activity. This notion is supported by the significant correlation between diaphragm motor unit fatigue resistance and the mean SDH activity of muscle unit fibers, that was reported by Enad *et al*., (1989). The significant increase in CS in combination with the reduction in the fatigue rate *in vitro* that was observed in the trained diaphragms of our study, supports this hypothesis.

Second, upregulation of SOD in our experiments confirms similar findings reported by Powers *et al*., (1994a). An exercise-training -induced up-regulation of antioxidant enzyme activity in the costal diaphragm enables the diaphragm to more efficiently eliminate the free radicals that formed during exercise due to an increased mitochondrial respiration. Addition of exogenous antioxidants to the *in vitro* bath, has been shown to slow the fall of force output in fatiguing muscle (Barclay and Hansel, 1991; Diaz and Clanton, 1992; Shindoh *et al*., 1990; Reid *et al*., 1992a; Supinski *et al*., 1991). A possible mechanism of action for the resulting reduction of oxygen free radicals due to an increase in the antioxidant enzymes, may be the attenuation of the changes in the functional properties of the SR that accompanies skeletal muscle fatigue and which affect Ca^{2+} release and uptake. Khawli and Reid (1994), postulated that antioxidants, by limiting free radicals, reverse the process of sulfhydryl oxidation which promotes calcium release from the SR by opening release channels and inhibiting reuptake pumps. Two possible sites of action are the histidyl residue associated with the ryanodine-sensitive calcium-channel protein, and the calcium dependent ATPase of the SR. Both these regulatory proteins of the SR can be modulated by sulfhydryl oxidation caused by free radicals triggering rapid calcium efflux from terminal cisternae and decreased calcium uptake. This SR dysfunction induced increase in myoplasmic calcium concentration and depletion of the SR calcium stores has been associated with muscle fatigue (Westerblad *et al*., 1991). An inhibition of free radical accumulation due to antioxidants would promote calcium sequestration by closing release channels and stimulating reuptake enabling therefore a better calcium homeostasis. The significant increase in SOD activity in combination with the fatigue sparing effect that was observed in this study, supports this hypothesis.

Third, an endurance training-induced decrease in the CSA of costal diaphragmatic fibers with no change in the capillary-to-fiber ratio, has consistently been reported in the literature (Gosselin *et al.*, 1992a; Green *et al.*, 1987; Green *et al.*, 1989; Tamaki 1987; Powers *et al.*, 1992a; Powers *et al.*, 1992b). This could improve the fiber's relative oxidative function (Powers *et al.*, 1992a; Powers *et al.*, 1992b), as well as, reduce the distance for diffusion of gases, metabolites, and/or substrates. This notion is supported by studies of the adult cat diaphragm performed by Enad *et al.* (1989). These authors reported that the more fatiguable diaphragm motor units, were composed of fibers with larger CSA and lower SDH activities. In contrast, fatigue resistant units, were composed of smaller fibers with higher SDH activities. The average fiber size of muscle units were correlated with their fatiguability.

In Vitro Shortening Velocity in Costal Diaphragm Strips

A second principal finding of the present study is that endurance training does not alter diaphragmatic V_{max}. This finding does not support our initial hypothesis which was based on recent studies, demonstrating that low intensity endurance running and low to moderate intensity endurance swimming, resulted in a significant decrease in the percentage of MHC IIb in the costal diaphragm (Sugiura *et al.*, 1992a; Sugiura *et al.*, 1990). Our results confirm this findings showing a significant decrease in the relative proportions of the type IIb MHC. In addition our data showed an increase in MHC I. To our knowledge this is the first report to document such an increase. These results demonstrate, that although the fast to slow shift observed in the trained diaphragm strips was statistically significant, it was not physiologically significant since it did not effect V_{max}. A logical explanation for this response may be the small magnitude of the overall fast to slow shift of the trained diaphragm, in combination with the unaltered MLC isoforms.

Since during *in vitro* field electrical stimulation, all muscle fibers are recruited simultaneously, the speed of shortening measured is attributed to all the fibers of the diaphragm strip. Despite the statistically significant 33.9% decrease in type IIb MHC and the 10.4% increase in MHC I in the trained diaphragms, the overall muscle shift from fast to slow was ~4%, a physiologically insufficient magnitude to alter V_{max}. The hydrolysis of ATP by the myofibrilar ATPase enzyme is thought to be the primary determinant of V_{max} (Close, 1972). Although myofibrillar ATPase activity was not measured in this study, based on the small overall shift in the MHC isoforms we postulate that it was probably not significantlly different between groups. However, recent reports indicate that under certain conditions, factors other than changes in myofibrillar ATPase activity may be responsible for the regulation of V_{max}.

Myosin light chains exist in slow and fast forms, and regulate the binding of actin and myosin (Fitzsimons and Hoh, 1981; Lowey *et al.*, 1979). Lowey (1993), have shown that complete removal of the MLC (regulatory and alkali) resulted in a dramatic reduction in the velocity of acto-myosin shortening, without altering the myosin ATPase activity. Sweeney *et al.* (1987), studied the Vmax of type IIb fibers that contained indistinguishable MHC but different alkali chain (LC1f/LC3f) ratio, and reported that the difference in V_{max} of the two groups of fibers was correlated with the alkali chain ratio. Further, data from skinned fibers treated to partially remove LC2, exhibit decreased V_{max} without altering isometric tension (Hofmann *et al.*, 1991; Moss, 1992). Therefore, the unaltered MLC isoform composition in the trained diaphragms, that was observed in the present study may be a possible factor, for the unchanged V_{max}. To our knowledge this is the first study to examine the effects of endurance training on the composition of the MLC isoforms of the costal diaphragm.

C-protein is known to be involved in the structure of the thick filament and may also play a role in the regulation of the mechanics of the cross-bridge stroke. Hofmann *et al.* (1991), reported that partial extraction of C-protein significantly increased Vmax at submaximal levels of calcium activation. The effects of exercise on C-protein are not known.

The results of the present study suggest that in addition to the small magnitude of the overall fast to slow shift in the MHC of the trained diaphragms, the lack of transformation of the MLC isoforms may be responsible for the unaltered Vmax after ten weeks of endurance training.

Specific Force in Costal Diaphragm Strips

The results of this study revealed that endurance training does not alter diaphragmatic specific force, and support therefore our initial hypothesis. In order for the maximal isometric specific tension of the diaphragm to be decreased, one of the following intrinsic mechanisms must be altered by endurance training: 1) the number of cross bridges in parallel per unit area; 2) the amount of force produced per cross bridge; 3) calcium handling during maximal activation (EC coupling); 4) a combination of these. None of these mechanisms seems to be altered during maximal force production of the unfatigued diaphragm, after a ten week endurance exercise program. This is reasonable, and consistant with the metabolic ceiling theory. The greater demand for ATP that is imposed on the muscle due to incresed metabolic load (exercise) is compensated by an increase in the mitochondrial volume. This mitochondrial increase plateus when adequate amounds of ATP can be supplied to the contractile apparatus. A further increase in mitochondria would not be usefull, since they would occupy more space, resulting in a contractile protein decrease and therefore a decrease in specific tension that would be accompanied by a decrease in ATP demand. The present study suggests that the already, highly oxidative diaphragm is metabolically plastic, but a metabolic ceiling prevents a decrease in contractile proteins and therefore a decrease in specific tension.

Summary and Conclusions

It has been shown that endurance training increases oxidative and antioxidant capacity of the costal diaphragm as well as capillary density. A fast to slow shift of the MHC isoforms of the diaphragm has also been noted to follow low intensity endurance exercise. The present study was designed to systematically examine the effects of these exercise induced cellular diaphragmatic adaptations in the fatigue rate, V_{max}, and specific force of the rat costal diaphragm. This was accomplished by examining these parameters in costal diaphragm strips from trained and control female Sprague-Dawley rats *in vitro*. Trained animals underwent a ten week endurance training program and their diaphragms exhibited significant increases in oxidative and antioxidant capacity as well as a fast to slow MHC isoform shift.

The results of this study show that training has a fatigue sparing effect on the diaphragm *in vitro*. Two electrical stimulation paradigms of different duration were used to induce muscle fatigue *in vitro*. Low frequency and duty cycle isometric contractions were used in both protocols. The trend of the trained diaphragms, to produce higher forces compared with controls after ten minutes of stimulation at the initial experiment, led us to postulate that the approximatelly 50% decrease in force production was representing fatigue of the fast fibers. This is logical, since all muscle fibers contract simultaneouslly *in vitro* and type IIb and IIdx comprise 60% of the diaphragm where type IIa and I comprise 40%. Knowing that our treatment (exercise) had effected predominantlly the type I and IIa diaphragm fibers, we conducted separate experiments using a sixty minute electrical stimulation paradigm, to discern differences between the fibers that had been adapted to exercise. Indeed, the repeat experiments demonstrated significant effects of the treatment, showing that the magnitude of the percent force reduction, between control and trained diaphragm strips, statistically differentiated after fifteen minutes of stimulation and remained different until the termination of the experiment. The specific cellular mechanisms for the training induced fatigue sparing effect were not the objective of this study and remain unknown. Possibly, the training induced increases in oxidative and antioxidant capacity and capillary density, prevent the SR dysfunction during fatigue by maintaining a better calcium homeostasis. This provides an interesting area for future research.

Another finding of this study is that diaphragmatic V_{max} is unaltered in the trained animals as compared to the controls, despite the decrease in the proportion of type IIb MHC and the increase in type I MHC in the trained diaphragm. We believe that the small magnitude of the overall fast to slow MHC shift did not significantly alter myofibrillar ATPase and thus V_{max}. This is speculative, however, since myofibrillar ATPase was not measured. Another possibility is that the unaltered MLC isoform composition that was observed in this study is responsible for the unchanged V_{max} since evidence exists that MLC play a paramaount role in muscle V_{max} regulation. This is very speculative and provides an interesting area for future research.

Finally, this study demonstrates that endurance exercise does not alter diaphragmatic specific force. This is consistant with the metabolic ceiling theory, since the exercise induced mitochondrial volume increase in the costal diaphragm, does not occur disproportionally to the contractile proteins and therefore does not effect P_O.

APPENDIX A
BIOCHEMICAL ASSAYS

Citrate Synthase

modified from: Srere (1969).

Principle:

$$\text{acetyl CoA} + \text{oxalacetate} + H_2O \xrightarrow{CS} \text{citrate} + \text{CoASH} + H^+$$

$$\text{CoASH} + \text{DTNB} \longrightarrow \text{mercaptide ion*}$$

*mercaptide ion mmol / cm extinction coefficient at 412 nm is 13.6

Solutions:

A. General buffer**
100mM potassium phosphate buffer

Vol in dH_2O	KH_2PO_4	K_2HPO_4
100 ml	0.4967 g	1.1060 g
500 ml	2.4836 g	5.5302 g
1000 ml	4.9672 g	11.0604 g

B. Standard homogenization buffer**
100mM potassium phosphate buffer with 0.05% BSA

Vol in dH_2O	KH_2PO_4	K_2HPO_4	0.05% BSA
100 ml	0.4967 g	1.1060 g	0.05 g
500 ml	2.4836 g	5.5302 g	0.25 g
1000 ml	4.9672 g	11.0604 g	0.50 g

C. Detergent protocol homogenization buffer**
10mM potassium phosphate buffer with 0.005% BSA

Vol dH_2O	Vol Soln B
90 ml	10 ml
900 ml	100 ml

D. Detergent protocol Triton X100 buffer**

10mM potassium phosphate buffer with 0.005% BSA and 0.01% Triton X100

Vol dH_2O	Vol Soln B	Triton X100
90 ml	10 ml	10 µl
900 ml	100 ml	100 µl

**Adjust pH to 7.4 with KOH. Make fresh weekly. Store at 0-5°C.

Reagents and Substrate Stocks:

1. Citrate synthase cocktail

DTNB (Ellman's Reagent: 5-5' dithio-bis 2-nitrobenzoic acid, Sigma # D-8130: FW = 396.4, store dry powder on shelf at room temperature), Triton X100, acetyl CoA (AcSCoA: Sigma # A-2772: FW = 809.4, store powder dessicated below 0°C), 10mM EDTA

Vol in Soln A	DTNB	Triton X100	AcSCoA	EDTA
50 ml	0.0021 g	35 µl	0.0043 g	0.1861 g
100 ml	0.0042 g	70 µl	0.0086 g	0.3722 g
200 ml	0.0084 g	140 µl	0.0152 g	0.7444 g

2. Oxalacetate cocktail

oxalacetate (OAA), Sigma # o-4126: FW = 132.1; store powder dessicated below 0°C; [stock] = 12.2 mM in buffer

Vol in Soln A	OAA
10 ml	0.0161 g
20 ml	0.0192 g

Procedure:

Tissue Preparation:

Homogenize skeletal muscle with ice-cold (0-5°C) potassium phosphate buffer. Use Solution B for standard homogenizations, Solution A for the control medium experiments, and a combination of Solutions C and D for the mechanical blender/detergent protocol. Final dilution for all homogenates should be 101/1.

Homogenate should be centrifuged (4°C) at 400 g (1575 rpm) for 10 min to remove cellular debris. The supernatant should be withdrawn and stored ice-cold until assay.

Assay Procedure:

Set spectrophotometer thermostat to 25°C or incubate reagents and homogenate at 25°C until equilibrium is reached.

Set spectrophotometer wavelength to 412 nm.

Add the following to a 1.0 ml cuvette:
1.) 1.725 ml Reagent 1

2.) 15 µl sample (45 µl with blender/detergent technique)

Wipe excess sample moisture from the outside of the pipette, dispense, and wash the pipette twice in the cuvette mixture. This will ensure that this small volume of viscous fluid can be dispensed accurately and reliably.
Wait 5 min - following this period deacylase activity levels are insignificant.

3.) 75 µl Reagent 2

Reaction commences when OAA is added. Vortex final mixture and read absorbance A412 from 1-4 min. Run samples in duplicate. The progress curve should be linear for at least 3 min when the rate does not exceed 0.100 A/min.

Calculation of Activity:

1.) Take linear portion of curve and determine [A412]/min.

2.) Calculate rate of citrate synthase in µmol/min by dividing [A412]/min by 13.6 (extinction coefficient for mercaptide ion).

3.) Determine activity per wet weight muscle be multiplying quotient in step 2 by final dilution factor (e.g., 101/1 x 1.21/0.01 = 12,221)

or µmol/g/min = [A412]/min x 1/E.C. x D.F.
µmol/g/min = [A412]/min x 1/13.6 x 12,221

Notes:

All solutions are stable for about 1 month when stored at - 70°C, except for OAA which should be made fresh daily. Potassium phosphate buffer can be stored at 0-5°C for about a week.

Superoxide Dismutase Assay

Reference: Sun and Zigman (1978).

Principle of operation:

Epinephrine undergoes autoxidation in air (or solution exposed to air) to form adrenochrome which absorbs light maximally at 320nm (must use quartz cuvets). SOD inhibits epinephrine oxidation by transfering O_2^- to H_2O_2. Therefore the amount of inhibition is in direct proportion to SOD activity.

Reagents:

1). 30 mM Epinephrine (FW = 333.3) in 0.1N HCl

EPI	Final volume(diH_2O)
0.01g	1
0.02g	2
0.2g	20

Note:

pH should be approximately 1.3 (important!)
Epi can be prepared and frozen in small aliquots for later use (freeze at -80°C)

2). NaCO3- (FW=84.01) buffer pH = 10.2 (with EDTA; FW = 372.2)

$NACO_2$	EDTA	Final volume (di H_2O)
.21	.00185	50
0.42	.0074	100

Procedure: (note: must use quartz cuvets due to wavelength)

To Run Blank:

1. Add 2 ml of NACO3 buffer to cuvet ;
add 20 µl Epi;
add 100µl H_2O
mix 4 times with vortex;
wait 5 min and read OD change over the next 3 minutes
blank should be 0.08-0.09 Od units/min

To Run Sample:

2. Add 2 ml of NACO3 buffer to cuvet ;
add 20 µl Epi;

add 100µl homogenate (100:1 dilution; 100 mM phosphate buffer)
mix 4 times with vortex;
wait 5 min and read OD change over the next 3 minutes

To Calculate Activity:

Amount of activity which causes 50% inhibition of Epi oxidation is equal to 1 unit; therefore activity = [(ΔOD.min sample- ΔOD Blank)/ΔOD.min blank] /0.5 x total dilution factor (2120) DF = 100/1 x 2.12/.1 = 2120

Glutathione Peroxidase (total)

modified from: Nakamura et al. (1974); Flohé and Günzler (1984).

Principle:

The glutathione peroxidase (EC 1.11.1.9) assay utilizes a coupled reaction of glutathione peroxidase with glutathione reductase. Glutathione peroxidase catalyzes the reaction below:

$$\mathrm{ROOH + 2GSH \xrightarrow{GPX} GSSG + ROH + H_2O}$$

Glutathione reductase catalyzes the following reaction:

$$\mathrm{GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+}$$

The rate of change in absorbance of NADPH per minute is followed @ 340 nm. One unit of glutathione peroxidase activity is defined as the amount required to oxidize 1 µmole of GSH/min corresponding to 0.5 µmole NADPH oxidized per minute.

Reagents:

1. Potassium phosphate - monobasic (KH_2PO_4): M.W. = 136.09 daltons

2. Potassium phosphate - dibasic (K_2HPO_4): M.W. = 174.18 daltons

3. Ethylenediaminetetraacetic acid (EDTA): F.W. = 372.2 daltons

4. t-butyl hydroperoxide (70% solution): M.W. = 90.1 daltons Sigma # B-2633

5. Glutathione reductase (155 U/mg) Sigma # G-4751

6. Glutathione-reduced (GSH): M.W. = 307.3 daltons
Sigma # G-4251

7. ß-nicotinamide adenine nucleotide phosphate - reduced form (NADPH) M.W. = 833.4 daltons Sigma # N-1630

Solutions:

A. 36.5/63.5 (monobasic/dibasic) mM potassium phosphate buffer with 10mM EDTA in dH_2O (no BSA). Adjust pH to 7.4 with KOH. Make fresh monthly; store at 0-5°C.

Vol in dH_2O	KH_2PO_4	K_2HPO_4	EDTA
100 ml	0.4967 g	1.1060g	0.3722 g
200 ml	0.9935 g	2.2121g	0.7444 g
500 ml	2.4836 g	5.5302g	1.8610 g

B. Reaction cocktail containing 0.2974 U/ml glutathione reductase1.25 mM GSH, and 0.1875 mM NADPH in Solution A. Make fresh daily; store at 0-5°C until ready for use.

Vol in buffer	GSH	NADPH	Glut. Reductase
25 ml	0.0110 g	0.0045 g	30 µl
35 ml	0.0154 g	0.0063 g	41 µl
50 ml	0.0220 g	0.0090 g	59 µl
100ml	0.0439 g	0.0179 g	118 µl

C. 12 mM t-butyl hydroperoxide in dH_2O. Make fresh daily.

Vol in dH_2O	t-butyl hydroperoxide
50 ml	78 µl
100 ml	155 µl

Procedure:

Tissue preparation:

Use skeletal muscle homogenate (1:20 wt/vol) further diluted 5 fold in ice-cold (0-5°C) 100 mM potassium phosphate buffer (pH = 7.4) w/ 10 mM EDTA and 0.05% bovine serum albumin (BSA) in dH_2O. Homogenate should be centrifuged (3°C) at 300 x g for 10 min to remove cellular debris. The supernatant should then be withdrawn and stored ice-cold until assay.

Assay procedure:

•set spectrophotometer thermostat to 25°C or incubate reagents and homogenate at 25°C until equilibration is reached.

•set spectrophotometer wavelength to 340 nm.

•add the following to 1 cm cuvette:

final vol = 1.5 ml		final vol = 2.0 ml
1) 1.2 ml reaction cocktail	or	1.6 ml reaction cocktail
2) 150 μl homogenate		200 μl homogenate

•incubate @ 25°C for ≥ 3 min.

3) 150 μl t-butyl hydroperoxide solution	200 μl t-butyl hydroperoxide solution

•reaction begins when the t-butyl hydroperoxide solution is added.

Mix by inversion and read absorbance A_{340} from 0-4 min. A blank made by substituting the homogenate with solution A with BSA in the above reaction procedure and should be run in triplicate prior to tissue homogenate analysis. The blank accounts for glutathione-independent reaction rate and should be subtracted from each sample's activity.

Calculation of activity:

1) Take linear portion of curve and determine $[A_{340}]$/min.

2) Calculate the rate of GSH oxidation (+ blank activity) in μmol/min by dividing $[A_{340}]$/min by 6.22 (extinction coefficient for NADPH) and multiplying by 2 (2 GSH:1 NADPH).

3) Determine activity (+ blank) by multipling quotient of step 2 by final dilution factor (e.g. 101/1 x 1.5/.15 = 1010)

4) Calculate blank activity.

5) Subtract blank activity from total activity per tube to obtain glutathione peroxidase activity.

overall:

μmol•g^{-1}•min^{-1} = [A_{340} x 1/E.C.x D.F.x moles GSH/NADPH) - blank activity

μmol•g^{-1}•min^{-1} = [A_{340} x 1/6.22 x 1010 x 2) - blank activity

APPENDIX B
QUANTITATIVE ISOLATION OF MYOFIBRILLAR PROTEIN

Quantitative isolation of Myofibrillar Protein

Reference: Solaro et al. (1971).

Assay Principle:

The insolubility of myofibrillar protein at normal pH and ionic strength is utilized to purify myofibrils via multiple buffer washes and centrifugations to remove all soluble proteins.

Note:

This technique does not separate connective tissue proteins from the myofibrils.

Reagents:

1. Sucrose
2. KCl
3. EDTA
4. Tris base
5. Triton X-100
6. Biuret protein reagents (see Total / C.T. protein assay)

Stock Solutions:

1. Sucrose buffer

250 mM Sucrose
100 mM KCl
5 mM EDTA
Adjust pH to 6.8

2. Triton buffer

175 mM KCl
0.5% Triton X-100
20 mM Tris
Adjust pH to 6.8

3. KCl buffer

175 mM KCl
20 mM Tris
Adjust pH to 7.0

Procedure:

1. Lable and weigh test tubes (13 x 100) for the samples.

2. Place weighed, minced muscle (100-200 mg) into glass homogenizing tube containing 4 ml of Sucrose buffer.

3. Homogenize on ice with glass on glass homogenizer until complete.

4. Transfer entire volume to pre-weighed test tube.

5. Centrifuge at 2500 x g, 5°C for 15 min.

6. Discard supernatent leaving a small amount to avoid disturbing the pellet.

7. Resuspend the pellet in 4 ml of Triton buffer and vortex well.

8. Centrifige at 2500 x g, 5°C for 10 min.

9. Repeat steps 6 - 8.

10. Discard supernatent, again leaving a small amount and resuspend in 4 ml of KCl buffer.

11. Centrifige at 2500 x g, 5°C for 10 min.

12. Repeat steps 10 and 11.

13. Discard supernatent leaving a small amount and resuspend in 0.5 to 1.0 ml KCl buffer depending on the starting muscle mass.

14. Weigh test tube with pellet and buffer.

15. Measure the protein concentration in the tube (see Total/C.T. protein assay)

Calculations:

1. Calculate the volume in the tube by subtracting the initial weight of test tube from the final weight. (this equals the volume in the tube assuming an approximate density of 1.0 g/ml)

2. Calculate the total amount of protein isolated from the sample (mg) by multiplying the protein concentration (mg/ml) times the total volume (ml).

3. Calculate the myofibrillar protein concentration (mg/g) of the sample by dividing myfibrillar protein (mg) by the initial wet mass of the sample (g).

APPENDIX C
SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Myosin Heavy Chain Procedure

Reference: Doucet and Trifaro (1988); Talmage and Roy (1993).

Gel Components:

The gels (separating and stack) are made from stock solutions stored at 4°C (SDS and Ammonium persulfate are stored at room temperature)

Separating Gel:	(8%)	(7%)
Stock Solutions:	Volumes for 2 slabs	
Glycerol (electrophoresis grade)	24.19 g (19.2 ml)	24.19 g
30%Acrylamide:Bis(50:1). Filtered. 29.41 g Acryl. 0.588 g Bis 100 ml dH_2O	17.068 ml	14.93 ml
1.5 M Tris (Base) (pH: 8.8)	8.53ml	8.53ml
Glycine (1 M) (do not pH)	6.4ml	6.4ml
10%SDS (store at room temp)	2.56ml	2.56ml
dH_2O	9.28 ml	11.42 ml
Mix well, vacuum, and add the following to initiate polymerization		
Temed	32 µl	32 µl
10% Ammonium persulfate	640 µl	640 µl
Total Volume	63.7 ml	63.7 ml

Over lay with 20% ethanol.

After setting wash off ethanol with 0.1%SDS, blot dry with filter paper and pour stacking gel

Stacking Gel:	(4%)
Solutions:	(Vol. for 2 slabs)
Glycerol	5.67 g
30%Acrylamide:Bis (50:1)	2 ml
0.5 M Tris (Hcl)(pH:6.7)	2.1 ml
100 mM EDTA (pH:7.0)	0.6 ml
10%SDS	0.6 ml
dH_2O	5.04 ml

Mix well, vacuum, and add the following to initiate polymerization

Temed	8 µl
10% Ammonium persulfate	150 µl
Total Volume	10 ml

Prepare 1.5 L upper running buffer, take 500 ml of it and use as an upper running buffer, dilute other 1 L with 1l dH_2O and use whole 2 L as a lower running buffer. Running buffers must be cold. Keep in refrigerator until use. Also, you can use cold dH20 to prepare running buffer.

Upper Running Buffer:

Tris (Base):	18.171 g
Glycine	16.89 g
SDS	1.5 g
dH_2O	1.5 L

Running conditions:

Set current up 0.12mV for 1 slab (0.24 for 2 slabs). Run under constant voltage for 20-24 hours. Voltage is going to be around 100V for 2 slabs

Myosin Light Chain Procedure

Reference: Doucet and Trifaro (1988).

Gel Components:

The gels (separating and stack) are made from stock solutions stored at 4C (SDS and Ammonium persulfate are stored at room temperature)

Separating Gel:	(12%)	(12%)
Stock Solutions:	Volumes for 1 big slab	~2 mini slabs
Glycerol (electrophoresis grade)	12.1 g	4.03 g
30%Acrylamide:Bis(50:1). Filtered. 29.41 g Acryl. 0.588 g Bis -------------- 100 ml dH_2O	12.8 ml	4.27 ml
1.5 M Tris (Base) (pH: 8.8)	4.27 ml	1.423 ml
Glycine (1 M) (do not pH)	3.2 ml	1.07 ml
10%SDS (store at room temp)	1.3 ml	427 µl
dH_2O	8.91ml	2.97 ml

Mix well, vacuum, and add the following to initiate polymerization

Temed	16 µl	5.3 µl
10% Ammonium persulfate	320 µl	106.7 µl
Total Volume	31.85 ml	10.62 ml

Over lay with 20% ethanol.

After setting wash off ethanol with 0.1%SDS, blot dry with filter paper and pour stacking gel

Stacking Gel:	(4%)	
Solutions	Vol. for 2 big slabs	for 1 or 2 mini slabs
Glycerol	5.67g	2.835g
30%Acrylamide:Bis (50:1)	2 ml	1 ml
0.5 M Tris (Hcl)(pH:6.7)	2.1 ml	1.05 ml
100 mM EDTA (pH:7.0)	0.6 ml	0.3 ml
10%SDS	0.6 ml	0.3 ml
dH_2O	5.04 ml	2.52 ml

Mix well, vacuum, and add the following to initiate polymerization

Temed	8 µl	4 µl
10% Ammonium persulfate	150 µl	75 µl
Total Volume	10 ml	5 ml

You can increase the amount of temed for stock solution and AP you can get faster polymerization (Even you can double it)

Running Buffer (pH:8.8, adjust pH with NaOH):

Tris (Base)	(:32.5 mM)	2.755 g
Glycine	(288 mM)	21.62 g
SDS	(0.1%)	0.7 g
-----------------------------------		
dH_2O		0.7 L

Running buffers must be cold. Keep in refrigerator until use.

Running conditions:

Set current up 50mV for 1 slab (100mV for 2 slabs). Run under constant current for approximately 40 min (monitor the gel, when colored line disappears wait 10 min more).

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BIOGRAPHICAL SKETCH

Ioannis S. Vrabas was born in the community of Kalamaria in Thessaloniki, Greece, on April 26, 1966. He graduated from the A' Lycee of Kalamaria in June, 1984. In July 1988, he completed a Bachelor of Science degree in physical education and exercise science at the Aristotelian University of Thessaloniki. An interest in the scientific basis of sport and exercise led him to the United States of America to pursue graduate studies in the field of exercise physiology. In 1990 he earned a Master of Science in Education degree in exercise and sport sciences at Queens College of the City University of New York under the direction of Drs. William McArdle and Michael Toner. Upon graduation, he accepted a doctoral student assistantship in the Department of Kinesiology at Louisiana State University in Baton Rouge, LA. In 1991, Ioannis continued his graduate work at the University of Florida in the Department of Exercise and Sport Sciences, under the direction of Drs. Stephen L. Dodd and Scott K. Powers, focusing on the adaptive biochemical and contractile responses of skeletal muscle to various stimuli including exercise training, pharmacological treatments, and ischemia. Upon receiving a Doctor of Philosophy degree in health and human performance with a minor in physiology, Ioannis will return to his homeland to pursue an academic career, after the completion of a two year mandatory naval service.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Health and Human Performance and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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